Identification and Characterization of Transformed Cells in Jaagsiekte, a Contagious Lung Tumour of Sheep

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CONTENTS

DECLARATION

I hereby declare that:

a) this thesis has been composed by myself

b) it has not been accepted in any previous application

c) the work has been carried out largely by myself. The subject of this thesis was part of a larger project on sheep pulmonary adenomatosis and therefore some of the work was performed in collaboration with colleagues at Moredun Research Institute. Where such work occurred, this fact is gladly acknowledged.

F.A. JASSIM

March, 1988

DEDICATION

I dedicate this thesis to my wife Lubab, and to my daughters, Aseel and Rana.

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ABBREVIATIONS

SPA	-	sheep pulmonary adenomatosis
SPARV	-	retrovirus of sheep pulmonary adenomatosis
RDDP	-	RNA-dependent-DNA polymerase
MPMV	-	Mason-Pfizer monkey virus
JRV	-	jaagsiekte retrovirus
x g	-	times gravity
PC	-	phosphatidylcholine
PG	-	phosphatidylglycerol
EGF	-	epidermal growth factor
ECM	-	Extracellular matrix
PBS	-	phosphate buffered saline
DMS	-	double minute chromosomes

ABSTRACT

Jaagsiekte is a contagious lung tumour of sheep in which two types of secretory epithelial cell in the lower respiratory tract are transformed. These cells are type II pneumocytes in the alveoli, and the cells of Clara in the terminal bronchioles.

An efficient and reproducible technique is described for the isolation of transformed SPA cells. It includes three basic steps: Prolonged trypsinisation to kill fibroblasts, magnetic removal of macrophages, and adherence to remove the rapidly adherent cells. The resultant preparations of lung cells were enriched to 96.6% type II pneumocytes.

Four cell lines were initiated from lungs of sheep with pulmonary adenomatosis and two of the cell lines have been continuously propagated *in vitro* for more than 140 passages. The morphologic and cytogenetic characteristics of these lines have been examined. The cells are epithelial and demonstrate ultrastructural features consistent with the tumour of origin. The lines are aneuploid and contain a spectrum of cytogenetic marker chromosomes.

SPARV P25 was detected in all cell lines in early passes, but was undetectable after 11th passage.

After 16 or 20 *in vitro* passages, three cell lines grew in soft agar with low cloning efficiency. However, the efficiency increased with higher passage numbers.

The effect of bromhexine HCl and prednisolone on the morphology and ultrastructure of SPA cell lines (JS7 and JS8) was studied. Cells treated with prednisolone lost their squamous epithelial shape and assumed a fusiform swirling appearance. Coincident with this change in morphology, the JS7 cells also lost the characteristic cytoplasmic lamellar bodies but not apical microvilli nor desmosomes.

Cells treated with bromhexine HCl showed an increase in the number and size of lamellar bodies, and retained their squamous epithelial appearance.

When the cell lines were transplanted subcutaneously in athymic nude mice, they induced palpable fluid-filled, cyst-like tumours at the site of injection. Tumour cells had a morphology similar to that of the parent cells and the original adenomatosis lung tumour cells of jaagsiekte, even though they had been through many in vitro passages. Ultrastructural and cytogenetic analysis of cell cultures derived from the nude mice tumours showed these to possess only the sheep karyotype.

SPA was successfully reproduced in neonatal lambs by intratracheal inoculation of passaged cells. Macroscopic lesions of SPA were observed in lambs and confirmed by histopathology and electron microscopy. Cell cultures were established from the lungs of these lambs. Replication of a retrovirus in these cultures was demonstrated by the detection in the supernate of SPARV

P25. The presence of the retrovirus in the lung fluids from these lambs was also demonstrated.

Thus for the first time permanent cell lines that contain the SPARV are available and should considerably facilitate further studies on this virus and the tumour it causes.

CHAPTER 1

REVIEW OF LITERATURE

Sheep pulmonary adenomatosis (SPA) is a contagious neoplasm of sheep and is the most common tumour in this species. Current interest in SPA (Synonyms: jaagsiekte; ovine pulmonary carcinoma) has been stimulated by its infectious character and the similarity of the lesions to those of human bronchioloalveolar carcinoma, which is believed to arise from the same cell types as in SPA. As well as its potential use as a model for human bronchioloalveolar carcinoma, SPA offers a convenient model of a transmissible mammalian adenocarcinoma. SPA is caused by a retrovirus which, apart from mouse mammary tumour virus, is the only retrovirus known to be involved in neoplastic transformation of epithelial cells.

In the following literature review, information supporting the statements above will be presented in three sections; a description of SPA disease comprising clinical signs, pathology and epidemiology; the features and functions of type II pneumocytes and Clara cells, both *in vivo* and *in vitro*; and evidence for the role of an incompletely described retrovirus as the causal agent of SPA.

1. DESCRIPTION OF SPA

A). Historical background

The name jaagsiekte is an Afrikaans term from "jaagt", to drive, and "ziekte", a sickness. It is so named either because affected animals look as if they have been driven, or because the disease was first noted when animals were moved from one place to another.

Although the disease has been known through anecdotal evidence since the early 18th century in South Africa (Tustin, 1969), the first description of jaagsiekte was published in 1904 by Robertson. Circumstantial evidence indicates that SPA originated in Spain and the disease was introduced to South Africa by Merino sheep imported from that country (Tustin, 1969; Wandera, 1970b).

Jaagsiekte has long been recorded in Britain. Lesions described in sheep lungs by Dykes and McFadyean (1888) and McFadyean (1894) were attributed to verminous pneumonia but, later, McFadyean (1920 & 1938) stated they were unmistakenly sheep pulmonary adenomatosis.

In Iceland a disease similar to jaagsiekte, and referred to as "epizootic adenomatosis", was described by Dungal et al (1938). It caused extensive losses among the sheep population and first appeared following the importation of infected rams from Germany in 1933. Environmental factors such as chill or sudden drop in temperature were thought to increase the fatality rates

and there was evidence that unaffected sheep contracted the disease if they came in contact with the affected animals.

B). Epidemiology

In the absence of a reliable diagnostic test, information on the epidemiology of jaagsiekte entirely relies on the clinical signs, gross pathology or histopathology of the tissue.

Distribution and prevalence

Pulmonary adenomatosis has been reported from many countries worldwide such as Bulgaria (Enchev et al, 1958), Canada (Stevenson et al, 1982), Czechoslovakia (Beseda, 1957), People's Republic of China (Deng et al, 1981), Chile (Schulz et al, 1965), East Germany (Jakob and Krause, 1965), France (Moraillon and Yalcin, 1967), Greece (Christodoulous and Tarlatzis, 1952), Iceland (Dungal et al, 1938), India (Damodaran, 1960), Iraq (Al-Zubaidy and Sokkar; 1979), Iran (Naghshineh and Sohrabi, 1972), Ireland (McCullough et al, 1985), Israel (Nobel, 1958), Italy (Romboli, 1959), Kenya (Shirlaw, 1959), Mexico (Eguiluz and De Aluja, 1981), Peru (Cuba-Caparo et al, 1961), Portugal (Madeira, 1949), Roumania (Adamesteanu et al, 1969), South Africa (Tustin, 1969), Spain (Dualde-Perez, 1963), Turkey (Akcay, 1956), United Kingdom (McFadyean, 1988), United States (Marsh, 1966), USSR (Mitrofanov, 1963), West Germany (Cohrs, 1966), Yugoslavia (Cvjetanovic and Martincic, 1962).

Though the disease has been reported to be endemic in most countries, its occurrence in others is considered to be sporadic (Verwoerd and Tustin, 1985).

According to Dungal et al (1938) the occurrence of the disease in Iceland resulted in the deaths of up to 50 to 80% of sheep in some flocks. The high incidence was attributed to the genetic susceptibility of Icelandic sheep to the disease, as well as the existing methods of sheep management such as folding together large numbers in autumn and close housing during winter. Although the infection spread very rapidly over much of the island the disease was eradicated completely by a strict slaughter policy (Sigurdsson, 1958).

In England, Blakemore and Bosworth (1941) described an outbreak of the disease in Norfolk in sheep brought from Scotland. However, in Scotland the disease was not confirmed until 1946 when Harbour and Jamieson described two outbreaks in Berwickshire and Aberdeenshire respectively. Subsequently, sheep pulmonary adenomatosis was detected in 223 sheep from various farms in Scotland during 1961-1971 (Nisbet, 1971; cited by Martin et al, 1979) and, more recently, Hunter and Munro (1983) showed that 24.6% of 1179 sheep lung specimens, examined over a 6-year period (1975-1981), showed lesions typical of SPA.

In South Africa, the incidence of jaagsiekte varies from less than 1% to as high as 20%, depending on breed susceptibility and management (Verwoerd and Tustin, 1985).

Although studies on materials obtained from abattoirs may not permit an accurate estimate of the disease incidence, a number of such reports suggest that jaagsiekte is of low incidence in many countries of the world, e.g. 0.1% in Peru (Cuba Caparo et al, 1961); 0.08% in Spain (Dualde-Perez, 1963); 0.1% in Chile; 0.2% in Northern Germany (Schulz et al, 1965); 0.87% in Turkey (Alibasoglu and Arda, 1975); 0.5% in Roumania (Baba et al, 1980), 0.25% in European Russia (Kostenko, 1964), and 0.04% in Yugoslavia (Nevjestic et al, 1971). However, in a similar study in Iran, Naghshineh and Sohrabi (1972) recorded an incidence of 8.2% although it is possible that this high figure could be due to the small number of specimens examined (120 samples).

Enchev et al (1958), emphasized the economic importance of SPA in Bulgaria and indicated that in affected flocks, losses reached 1% to 3%; whereas 4 years later, the disease occurred in epidemic form and caused losses of 35.5% on some farms (Enchev, 1961a). Losses of 10% to 30% of the affected flocks were reported in Greece (Christodoulous and Tarlatzis, 1952) and 30% annual mortality from jaagsiekte on some sheep farms was reported in Kenya (Shirlaw, 1959). In Russia, in the province of Azerbaijan, Aliev (1967) recorded 1.6 to 7.5% incidence of SPA.

Breeds affected

Whatever the differences of opinion are regarding breed and family predisposition to susceptibility, there is no doubt that the disease occurs in many different breeds of sheep such as Karakul (Dungal, 1946), Masai (Shirlaw, 1959), Merino (Mettam, 1927) and Awassi (Perk et al, 1971; AL-Zubaidy and Sokkar, 1979). Some investigations indicate that the Gottorp breed of Icelandic sheep and British breeds such as Greyface, Blackface, Halfbred and Cheviot are highly susceptible to SPA (Dungal et al, 1938; Martin et al, 1979; Hunter and Munro, 1983) , whereas other breeds are considered to be more resistant (Tustin, 1969).

Sex

Reports regarding male and female predisposition to SPA are conflicting. Some authors consider that the disease occurs equally in both sexes (Damodaran, 1960; Tustin, 1969) whereas others including De Kock (1929) and Kostenko (1968) are of the opinion that rams are more susceptible than ewes. In this connection it is of interest that Mackay and Nisbet (1966) attributed the higher incidence in rams partly to the fact that they are commonly housed and intensively managed, and partly to the fact that they usually live long enough to be exposed to the risk of contracting the disease, when compared with lambs or wethers.

Species affected

Pulmonary adenomatosis has been observed in no species of animals other than sheep and goats. From the literature, there is good evidence that SPA can also occur in goats. For example, Cuba-Caparo et al (1961) first described SPA in goats and showed that the gross and microscopic features of pulmonary adenomatosis lesions were very similar to those of SPA in sheep. Similar lesions have been reported by other workers (Rajya and Singh, 1964; Aliev, 1967; Tiwari and Pandit, 1967). In contrast, field cases of pulmonary adenomatosis in goats have not been reported in Iceland (Dungal et al, 1938), or in Scotland (Martin et al, 1979) despite the fact that the disease has frequently been described in sheep. Reports of the disease in goats have been questioned by Tustin (1969) who considers that they have arisen from inaccuracy of histopathological differentiation between epithelialization of the alveoli and adenomatosis.

On the other hand there is increasing evidence that under experimental conditions SPA can be transmitted successfully to goats with cell-free filtrates or lung fluids obtained from sheep with jaagsiekte (Sharma et al, 1975; Sharp et al, 1986). Two of 25 goats inoculated with seitz-filtered tumour material revealed typical SPA lesions on microscopic examination (Sharma et al, 1975).

Sharp et al (1986) also reported successful

transmission of the disease to goats. They inoculated three one-day-old goats intratracheally with lung fluid from infected sheep, which contained the retrovirus that causes SPA. Although no clinical signs were seen during the six-month period of the experiment, the lesions observed in one goat were similar to those described for pulmonary adenomatosis in sheep. The authors pointed out that the efficiency of transmission was very low compared with the transmission in newly born lambs and their observations, together with field reports by other workers (Cuba-Caparo, 1961; Cuba-Caparo et al, 1961; Rajya and Singh, 1964; Tiwari and Pandit, 1967; Aliev, 1967), underline the fact that the incidence of pulmonary adenomatosis in goats is very low.

Susceptibility of laboratory animals

Various attempts at transmission of SPA to laboratory animals with SPA tumour, tumour homogenate or cultured SPA cells have been carried out by several investigators.

Successful transmission of SPA to laboratory animals has not been reported except when tumour cells were inoculated into nude mice.

In 1959, Shirlaw claimed to have induced lesions similar to those of jaagsiekte in the lungs of rabbits inoculated intravenously with fragments of lung tumour, but failed to produce similar lesions in mice

inoculated intratracheally with tumour material. However, the results of this study are dubious, since repeated attempts to initiate infection in rabbits, guinea pigs, hamsters and rats, have failed (Dungal et al, 1938; Martin et al, 1979; Coetzee et al, 1976).

Transplantation of SPA tumour to nude mice has, however, been obtained, either with cultured cells derived from SPA tumour or with trypsin-disaggregated tumour tissue (Verwoerd et al, 1977; Zimber et al, 1984). In both studies, cystic tumours lined by a single layer of epithelial cells developed following inoculation of 10^7 cells subcutaneously. However, attempts to initiate cultures from these tumours for biological studies met with failure (Verwoerd et al, 1977).

C). Clinical signs

There is general agreement that sheep may possess typical lesions of jaagsiekte without showing noticeable symptoms (Cowdry and Marsh, 1927). Indeed, signs in affected sheep are only seen when the tumours become large enough to affect normal lung physiology and function (Martin et al, 1979; Sharp and Martin, 1983).

The incubation period of jaagsiekte is long and, although it cannot be defined precisely, it is generally regarded as months or even years. Because the onset of the disease is insidious, the clinical signs are mainly seen in adult sheep. The first symptom to appear

is a marked shallow and accelerated respiration accompanied by loss of weight. The signs become more aggravated as the disease advances and, if the animal is driven for any distance, it starts panting for breath, heaving at the flanks and finally lies down completely exhausted. In advanced cases, moist rales can be detected on auscultation. These result from fluid accumulation in the lungs, believed to be secreted by the transformed cells. When the head of the affected sheep is lowered and the hind legs are raised, the accumulated fluid pours out of the nostrils. This is regarded as a field diagnostic test (Dungal et al, 1938; Stevens, 1957). In some cases, although the rales are detectable, fluid is absent and such animals, when examined, have been found to have fibrosed lesions (Sharp, 1981).

The amount of "lung fluid" that is secreted varies considerably and in some instances up to 500 ml may be obtained daily from a single animal. The fluid is copious, milky, and contains aggregates of mucus and cells, of which macrophages are the predominent cell type.

There is no rise of temperature, except in the terminal stages of the disease when secondary infection intervenes. Appetite is maintained though loss of weight is obvious. The disease ends with the death of the animal, often as a result of secondary infection with Pasteurella haemolytica.

D). Pathology

Necropsy findings

Necropsy examination shows that both lungs are usually involved. In the initial stages of the disease the most striking features are small, round, greyish-white nodules of various sizes slightly protruding from the surface. The nodules may remain isolated or become confluent (Dungal et al, 1938; Cuba-Caparo et al, 1961; Wandera, 1971). In advanced and uncomplicated cases, the lungs become greatly enlarged and may reach three to four times normal size (Fig.1.1). The affected tissue is smooth, slippery and firm, and the cut surface of the tumour has a glistening, granular appearance, pink-grey in colour (Fig. 1.2), from which a frothy fluid oozes. The trachea, bronchi and bronchioles contain white frothy fluid which pours out of the trachea when the affected lung is kept on a flat surface (Fig. 1.1). In long standing cases the affected parts become firmer and harder to the touch, as a result of fibrosis.

Microscopic pathology

Histologically, SPA consists of nodular or sometimes diffuse lesions composed of alveoli or bronchioles lined by a single layer of cuboidal or columnar epithelial cells. Groups of proliferating cells arise at certain points to form papillary ingrowths projecting into the alveolar or bronchiolar lumina (Fig. 1.3). The tumour polyps are supported by varying

Fig. 1.1

Lateral aspect of lung from adult sheep with SPA tumour. Note diffuse consolidation in apical, cardiac and diaphragmatic lobes. Frothy and milky-coloured fluid oozes from the trachea (arrow).

Fig. 1.2

Cross section of the lung shown in Fig. 1.1 showing the granular appearance of lesions from the cut surface.

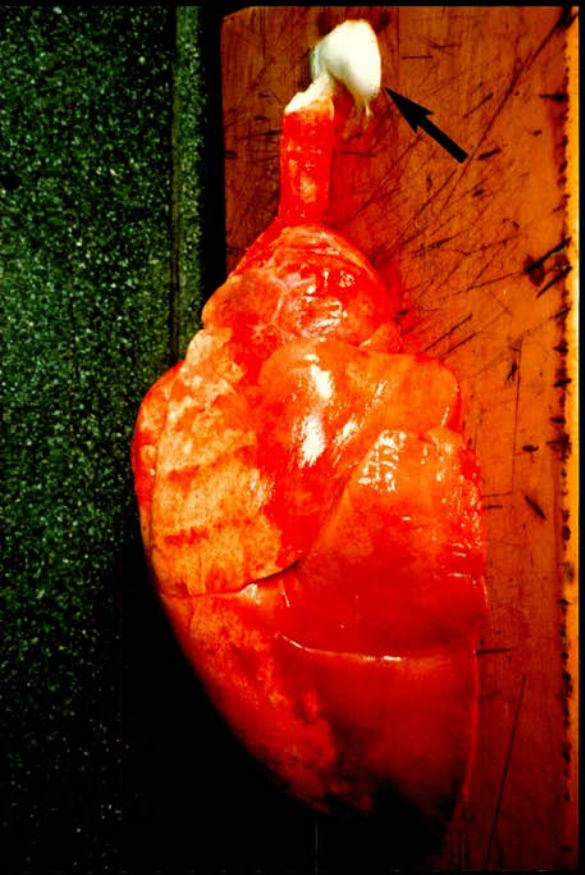
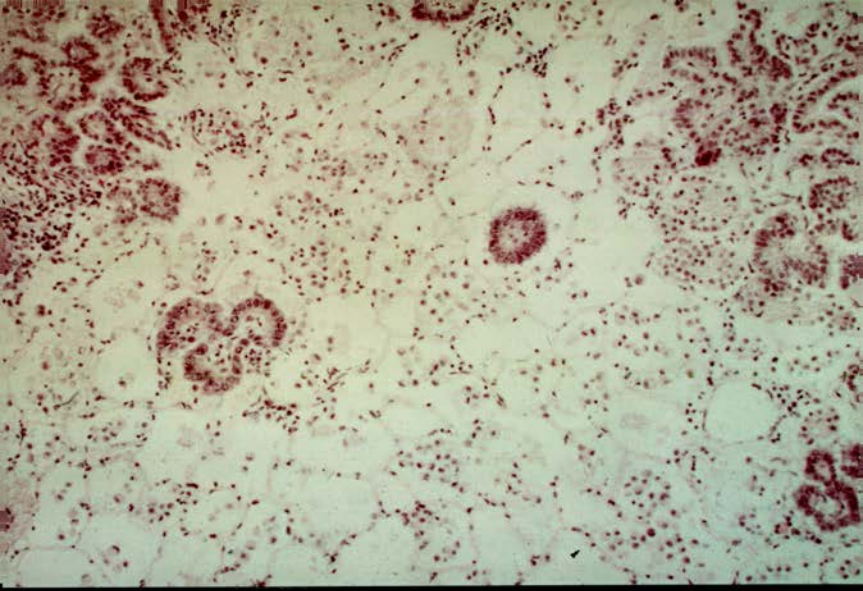
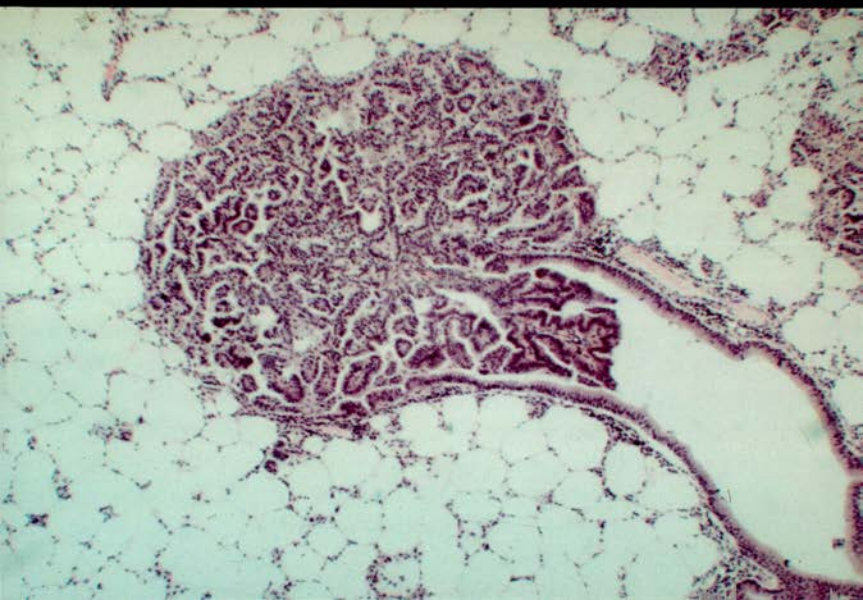


Fig. 1.3A,B

A). Section of sheep lung showing intra-alveolar proliferation and papilliform ingrowths arising from their epithelial lining. H & E, x 60.

B). Section of sheep lung showing a neoplastic focus in the alveoli extending into a bronchiole. H & E, x 150.





amounts of connective tissue stroma, depending on the age of the tumour (Dungal et al, 1938; Cuba-Caparo et al, 1961; Damodaran, 1960; Nobel et al, 1968; Al-Zubaidy and Sokker, 1979; Cutlip and Young, 1982). The fibrous stroma in the older jaagsiekte lesions leads to the formation of bizarre cystic or cyst-adenomatous lesions (Perk, 1982; Sharp and Angus, 1985). A macrophage response is an invariable feature in the extensive SPA lesions. Abundant macrophage cells are found in the alveolar spaces or surrounding the primary lesions.

The neoplastic nature of pulmonary adenomatosis in sheep was described for the first time by Aynaud, (1926). He found a bronchiolar lymph node metastasis in one case. Later, Dungal (1946) examined Aynaud's slides and confirmed his interpretations. Since then many reports of metastatic lesions confined to the regional lymph nodes have been recorded (Cuba-Caparo, 1961; Enchev, 1961b, 1962, 1963; Stamp and Nisbet, 1963; Santiago-Luque, 1963; Markson and Terlecki, 1964; Mitrofanov, 1964; Martincic and Cvjetanovic, 1967; Nobel et al, 1968 & 1969; Tustin, 1969; Wandera, 1967 & 1971; Sharma et al, 1975; Giesel and Bomhard, 1977; Baba et al, 1980; Cutlip and Young, 1982). The presence of metastasizing epithelial cells in afferent lymphatics suggested the lymphatic route of spread of SPA lesion to regional lymph nodes as reported by Enchev (1963). Extrathoracic metastases have also been reported (Enchev, 1963; Martincic and Cvjetanovic, 1967; Nobel et al, 1968; Tustin, 1969).

Considering the cell of origin, and the metastatic potential of the neoplasm, the terms "Malignant pulmonary tumour", "Pulmonary carcinomatosis", "Pulmonary carcinoma" or " Bronchioloalveolar cell carcinoma" have been proposed as more accurate terms and to replace the current nomenclature of jaagsiekte or SPA (Enchev, 1963; Tustin, 1969; Nobel et al, 1969; Hod et al, 1977; Nobel and Perk, 1978).

2. FEATURES AND FUNCTIONS OF TYPE II PNEUMOCYTES AND CLARA CELLS

A). Type II pneumocyte and Clara cell in normal lung

Type II pneumocyte

Histological and electron microscopic studies have shown that the normal mammalian alveolar epithelium consists of two morphologically and functionally distinct cell types. These are the type I pneumocyte (synonyms, type A pneumocyte, small alveolar cell, membranous pneumocyte; Sorokin, 1967), a squamous cell which covers most of the alveolar surface by its attenuated cytoplasm and type II pneumocyte (synonyms, type B pneumocyte, great alveolar cell, corner cell, niche cell, granular pneumocyte) a cuboidal cell, which is usually found in the corner of an alveolus (Wang et al, 1971; Mason et al, 1977).

It has been estimated that type II cells normally account for no more than 14% of rat lung cells and 16% of human lung cells (Crapo et al, 1982). The diameter of the cell ranges from 7μm to 14μm and the spherical vesicular nucleus is frequently eccentric. The Golgi apparatus, multivesicular bodies and rough endoplasmic reticulum are well developed. The cell is covered by short microvilli. This cell contains characteristic osmiophilic lamellar inclusions that are bound by a limiting membrane (Sorokin, 1967) (Fig. 1.4). While the size, shape and location of the lamellar bodies

Fig. 1.4

Transmission electron micrograph of a portion from normal sheep alveolus. Cells feature many microvilli (Mv), desmosomes (large arrows), and lamellar bodies (small arrows) the characteristic feature of type II pneumocytes. x 18000


MV

are similar in most species, the appearance of their contents, as seen by the electron microscope, varies. In the rabbit and sheep, the contents of lamellar bodies are not particularly osmiophilic (Kikkawa et al, 1968). However, in most species the inclusion bodies contain a highly osmiophilic material arranged in lamellae or whorls (Balis and Conen, 1964; Balis et al, 1966; Karrer, 1956; Sorokin, 1967; Kikkawa and Spitzer,1969). The amount of nonlamellar material seems to decrease as the lamellar bodies mature (Kuhn, 1968; Goldfischer et al, 1968). Lamellar bodies are secreted from the cell by exocytosis (Ryan et al, 1975) and Kliewer et al (1985) indicated that the limiting membrane of an apically located lamellar body may be fused with the plasma membrane. In consequence, a pore between the interior of the lamellar body and the alveolar lumen is formed, and the content is released. It has been estimated that a type II cell contains an average of 150 lamellar bodies, and an average of 15 lamellar bodies are secreted each hour by each cell (Young et al, 1982; Kliewer et al, 1985).

It has been suggested, on the basis of developmental and biochemical evidence, that the lamellar bodies are the sites where pulmonary surfactant is stored prior to its release from the cell to the alveolar surface (Kikkawa et al, 1968; Gil and Reiss, 1973). Nevertheless, it is not known yet whether they also participate in the biosynthesis or modification of phospolipids. There are suggestions that lamellar bodies

are at least involved in the synthesis of phosphatidyl-glycerol (Benson, 1980).

The structural precursors of the lamellar bodies of type II pneumocytes are not known, although many investigators believe that lamellar bodies in type II alveolar cells arise from the multivesicular bodies (Sorokin, 1967; Kikkawa and spitzer, 1969). According to this view, the product synthesized in the endoplasmic reticulum reaches the Golgi apparatus. The pinched-off vesicles then form multivesicular bodies, which finally become lamellar bodies (Collet and Chevalier, 1977).

However, alternative origins have been suggested by others. For example Klaus et al (1962) and Pattle (1972) consider the mitochondria as a source of lamellar bodies whereas Flaks and Flaks (1972) have suggested that both multivesicular bodies and mitochondria may be the precursors.

Several procedures for the isolation of lamellar bodies from several species of animals have been promulgated in the past few years (Williams et al, 1971; Hoffman, 1972; Gil and Riess, 1973; Valdivia, 1973; Hallman et al, 1976; Duck-Chong, 1978; Oulton et al, 1980; Sanders et al, 1980). The commonest approach is centrifugation over variously constructed sucrose density gradients.

The notion that the type II alveolar cell is the site of surfactant-lipid synthesis came in 1954, when Macklin using light microscopy was able to distinguish the

surfactant layer in the alveolus. He postulated that this alveolar lining layer arose from discrete organelles, later identified as lamellar bodies. Macklin's view is now widely accepted since the presence of normal surfactant activity in fetal lung correlates well with the concurrent appearance of type II inclusion bodies in most species. For example, lamellar bodies did not appear in developing mouse lung until the 18th day of gestation; this was also the time when surface-tension-lowering material appeared in the fetal lung (Buckingham and Avery, 1962). This general relationship was also shown to be true for the fetal rabbit (Kikkawa et al, 1968). An increase in the concentration of surface active dipalmityl lecithin in fetal rabbits occurred concurrently with a rapid increase in the number of lamellar bodies in type II cells. Similarly normal lamellar inclusion bodies appeared in the fetal lamb at 121 days of gestation and the normal surfactant activity developed about five days later (Kikkawa et al, 1965). This study also showed that the lamellar bodies contained phospholipids and that they were the precursors of the alveolar lining layer. The simultaneous appearance of lamellar bodies and surfactant was also found in the lung of fetal sheep by Orzalesi et al (1965).

In the same way that reduced surface tension is associated with the appearance of lamellar bodies, so does increasing surface tension correlate with reduced or abnormal lamellar bodies. Klaus et al (1962) noted in

vagotomized guinea-pigs that the numbers of lamellar bodies per type II cell were decreased and that a rise in the pulmonary surface tension paralleled the decrease in the number of lamellar bodies. They also suggested that the lamellar bodies are the intracellular source of surfactant. Also a morphologic change in the lamellar bodies was accompanied by an increase in pulmonary surface tension (Goldenberg et al, 1967). Schaefer et al (1964) found that guinea-pigs subjected to an atmosphere containing 15% carbon dioxide developed abnormal lamellar bodies and had significantly increased surface tension. The return to normal surface tension was accompanied by the appearance of normal lamellar bodies. Lamellar bodies are also greatly decreased in number or even absent in hyaline membrane disease or respiratory distress of the newborn human infants or lambs (Kikkawa et al, 1965).

The conclusions, derived from the above morphologic studies, that the lamellar body in the type II pneumocytes is involved in the production of surfactant material in the lung has been supported by direct analysis of the contents of lamellar bodies.

Kikkawa et al (1970), using a chlorofom/ methanol extraction technique, successfully prepared phospolipids from the surfactant layer as well as the lamellar bodies and demonstrated a similarity in composition between the two. They also found that phospholipids are the predominant constituents of the surfactant in both the lamellar bodies and the surfactant

layer. This view also was corroborated by autoradiographic studies (Faulkner, 1969; Askin and Kuhn, 1971; Chevalier and Collet, 1972).

Biochemical analyses indicate that the surfactant is a complex mixture of phospholopids, neutral lipids, proteins and, possibly, carbohydrates. The predominant components of surfactant are phospatidylcholine (PC) and phosphatidylglycerol (PG), which represent 71% and 5.4% of the total phospholipids respectively (Harwoord et al, 1975).

In addition to producing surfactant, the type II cell is also known to function as a reserve cell from which regeneration of type I pneumocytes occurs following acute alveolar damage (Evans et al, 1975; Adamson and Bowden, 1974; Gail and Lenfant, 1983). The type II cell, during the time of normal maturation, changes from a glycogen-laden epithelial cell (immature) to a cell which contains lamellar bodies (Kikkawa et al, 1968).

Clara cell

This cell has not been as intensively studied as the type II pneumocyte and its role remains unclear. The nonciliated columnar epithelial cells, named Clara after the description by Max Clara (1937), are located in the distal portion of the bronchioles and also in bronchi and trachea (Breeze and Wheeldon, 1977; Plopper et al, 1983). The Clara cells contain intracytoplasmic,

dense, non-lamellated, membrane-bound secretory granules and feature a well developed system of endoplasmic reticulum and Golgi apparatus. Although the Clara cell is non-ciliated, its apical surface does feature numerous microvilli. Additionally several studies have suggested that Clara cells are the stem cells of the cuboidal bronchiolar epithelium (Evans et al, 1978; Brody et al, 1987). Other studies have concluded that, based on morphological features and high levels of oxidative activity, the Clara cell is metabolically a very active cell (Azzopardi and and Thurlbeck, 1969; Cutz and Conen, 1971; Etherton et al, 1973). However, neither the secretory function of the Clara cell nor its mechanism have been established.

Most investigators agree that the Clara cell has a secretory function, and morphological evidence of this has been presented. Etherton et al (1973) observed decapitation of the apical cytoplasm of the cells and speculated that this represented the secretory mechanism of Clara cell.

Tritiated palmitate has been localized in Clara cells by autoradiography a short time after injection into animals, and at later time in the airways, suggesting a secretory process (Niden, 1967; Etherton et al, 1973).

The nature of the granules within the Clara cells has been studied with conflicting results. On the basis of histochemical staining and incorporation of

tritiated palmitate into the granules, Niden (1967) suggested that they contain phospholipid and that the Clara cell was the site of surfactant synthesis. This suggestion was supported by Azzopardi and Thurlbeck 1967 & 1969) who showed that the Clara cell was rich in choline-containing lipids, which were present as a lipoprotein complex. Cutz and Conen (1971) detected phospholipid in the granules, and their lability to pepsin digestion and the failure of lipid solvents to alter their electron microscopic appearance, suggested that protein is a major component of granules and the phospholipid is firmly bound to protein. These findings agree with those of Azzopardi and Thurlbeck (1969) and are supported by studies of Petrik and Collet (1974) who found incorporation of radioactive precursors of both lipid and protein into the granules. For example, tritiated choline chloride, a precursor of phosphatidylcholine (lecithin), was incorporated into granules, as well as tritiated galactose. All of the above studies have led to the notion that the granules in Clara cells are mainly proteinaceous, secretory and contribute to the noncellular lining of the lower respiratory tract.

B). Cells in SPA tumour

Transmission electron microscopy has led to the view that SPA is derived mainly from transformed type II pneumocytes and to a lesser extent from Clara cells.

The tumour cells in the alveolar lesion possess features consistent with the characteristics of type II pneumocytes such as microvilli, desmosomes, masses of glycogen granules and lamellar bodies (Perk et al, 1971; Nisbet et al, 1971; Bucciarelli, 1973; Cutlip and Young, 1982). The intrabronchiolar neoplasms consist of non-ciliated epithelial cells that have ultrastructural features of secretory cells and contain numerous mitochondria and electron-dense granules in their cytoplasm with the features of non-ciliated bronchiolar (Clara) cells (Nisbet et al, 1971; Nobel and Perk, 1978). The SPA tumour mass consists of cells exhibiting different degrees of cell differentiation and organization (Hod et al, 1974). In the early tumour lesion, the neoplastic cells contain numerous microtubules which are absent in advanced lesions. In contrast, tumour cells in advanced lesions contained huge accumulations of glycogen with or without cytosomes. On the basis of histological and ultrastructural resemblance of SPA to human bronchioalveolar carcinoma, SPA was proposed as a valuable animal model for studying this type of human tumour (Nisbet et al, 1971; Nobel and Perk, 1978).

C). Type II and Clara cells in culture

Reliable techniques for the extraction and purification of type II cells have been documented and short-term cell cultures of type II alveolar cells from a variety of animals have been established successfully (Kikkawa and Yoneda, 1974; Mason et al, 1975, 1977a;

Frosolono et al, 1976; Dobbs and Mason, 1977; Greenleaf et al, 1979; Mettler et al, 1981; Saito et al, 1985).

Several methods are available to identify type II alveolar cells. The most specific method is electron microscopy by which lamellar bodies, the hallmark of type II cells, can be identified. However, because electron microscopy is a time consuming technique, several other methods have been developed for faster identification of this cell type; though they are not absolutely specific: for example, phase, fluorescent and light microscopy. Once type II cells adhere to a flask and flatten, they can be easily identified by phase contrast micoscopy where the intracellular inclusions can be seen as dark granules around the nucleus (McMahon et al, (1986). However, phase contrast microscopy cannot be used to diferentiate type II alveolar cells from macrophages in suspension, but they can be differentiated by fluorescent microscopy. Phosphine 3R is a fluorescent dye that partitions into lamellar bodies and can produce intense fluorescence (Mason et al, 1976). Acridine orange is another fluorescent stain used to identify type II alveolar cells. Cells stained by acridine orange showed intense orange fluorescence in the lipid-containing granules. The cells can also be identified by staining directly in culture after fixation with glutaraldehyde with a tannic acid and polychrome stain that shows both the granules and cell outlines (Robinson et al, 1984).

This stain can also discriminate between type II cells and other contaminating cells. Type II alveolar cells can also be identified by light microscopy after staining by the modified Papanicolaou stain (Kikkawa et al, 1975). With this stain lamellar bodies are seen as dark blue granules in the cell cytoplasm.

In certain cases, the type II alveolar cells isolated from rabbits and rats have been maintained in culture for relatively short periods of 1-2 weeks (Diglio and Kikkawa, 1977). In these conditions, the cells apparently lose both their morphological characteristics and proliferative potential. Frosolono et al (1976) claimed to have been able to maintain cultured type II cells isolated from adult rats and rabbits for over 1 year in vitro, with the retention of type II cell ultrastructural characterstics.

In addition to primary cultures of alveolar type II cells, several normal and transformed cell lines have been established that retain several characteristics of type II pneumocytes (Douglas and Kaighn, 1974; Kniazeff et al, 1976). For example, the AK cell line from foetal cat lung is characterised by its epithelial morphology and dense cytoplasmic granularity (Kniazeff et al, 1976). Ultrastructural analysis shows the presence of lamellar bodies, which occur in these cells through the 40th passage. Unfortunately this cell line appears to lack long-term in vitro stability, since the authors have indicated the development of abnormal karyotype in various clones at relatively early passages.

Transformed cell lines that retain type II characteristics for an extended period of time *in vitro* have been established from mouse and human sources (Stoner *et al*, 1975; Lieber *et al*, 1976). Line LA-4, a clone derived from urethane-induced mouse lung adenoma, was reported to contain lamellar bodies similar in structure to those observed in adenoma cells *in situ* (Stoner *et al*, 1975). Cell line A549 (Lieber *et al*, 1976) was initiated from a human pulmonary adenocarcinoma and has been found to have morphological characteristics similar to alveolar type II cells. This line will also synthesize disaturated phosphatidylcholine (DPPC) (Smith, 1977), produce colonies in soft agar, and tumours in mice. However, according to Mason and Williams (1977) the DPPC content from A549 cell line is very low and accounts for only 8.2% of total phospholipids from this cell line as opposed to 32% from freshly isolated type II alveolar cells. This figure is similar to the disaturated phospholipid concentration in fibroblasts and, for this reason, Mason and Williams (1977) dispute the designation of A549 cells as a cell line that retains the differentiated function expressed by epithelial cells *in vivo*.

Insofar as the Clara cell is concerned, although isolation procedures have been described, reports of *in vitro* propagation of this cell type are limited (Devereux, 1984; Aune *et al*, 1985). However, a report by Haugen and Aune (1986) describes the isolation and

purification of Clara cells from rabbit lung, with 85% purity, and their successful establishment in culture. Although the cells were found to have a low attachment efficiency (20%), and long doubling time (three to four days), the authors were able to maintain the cells for up to five passages in vitro. More recently, Clara cells were isolated from rabbit lungs using a procedure involving a continuous Percoll density gradient and elutriation. The cell preparations obtained by this method contained between 80% to 85% Clara cells (Brody et al, 1987).

Clara cells were identified by Nitroblue tetrazolium histochemical staining and electron microscopy (Devereux and Fouts, 1980).

3. CAUSAL AGENT OF SHEEP PULMONARY ADENOMATOSIS

A). Transmission

Back in 1904, Robertson reported unsuccessful attempts to transmit the disease from infected to healthy sheep by cohabitation and by inoculation with blood and affected tissues. Nevertheless, the thought that pulmonary adenomatosis was a transmissible disease was put forward by De Kock (1929), who also conducted transmission experiments. Although his findings were not convincing, as SPA lesions were detected in only one healthy sheep, De Kock's observations provided the first experimental indication of the infectious nature of the disease and signalled the start of intensified work to elucidate the aetiology of jaagsiekte.

In 1938, Dungal et al repeated the contact experiments of De Kock (1929) and successfully transmitted the disease after housing together healthy and infected sheep. Of the eight in-contact sheep, six showed lesions of pulmonary adenomatosis, but no clinical signs. In another experiment, one of three sheep given an intrapulmonary inoculation developed clinical signs eight months later, but signs of the disease were not reproduced in sheep given a filtered preparation. This result provided the first evidence for transmission of the disease by parenteral inoculation with suspensions containing the infectious agent.

The transmission experiments of Dungal (1946) conducted between 1939-41, provided the most elaborate studies so far undertaken to demonstrate the transmissibility of SPA. In this series of experiments neither feeding healthy sheep with faeces from infected ones, nor parenteral inoculation of blood was successful. However the disease was transmitted when sick sheep were kept in head-to-head contact with healthy sheep but not when the heads were kept apart. The infectious nature of exhaled air was also confirmed in these experiments when the expired air from an infected sheep, collected in a glycerine mixture, was used as inoculum by the intratracheal and intrapulmonary routes. When sheep were killed for examination, lesions were present some four to eight months after inoculation. Dungal also concluded that a pneumotropic virus was the cause of the disease and that the virus was confined to the lungs and bronchi and was present in expired air.

In 1959, Shirlaw claimed a highly successful transmission rate of sheep pulmonary adenomatosis since lesions similar to jaagsiekte, known in Kenya as "Laikipia lung disease", were found in nine of 11 sheep given fragments of lung material intravenously. However, these results were not convincing because Laikipia lung disease is generally considered to be a complex of chronic pneumonias, such as maedi, SPA and bacterial infections. Furthermore, Markson and Terlecki (1964) indicated that they found no evidence of SPA lesions in sections of lungs

from the experimental lambs sent for confirmation by Shirlaw, although sections of lungs from field cases of Laikipia lung disease showed lesions typical of jaagsiekte.

Transmission studies with cell-free or ultrasonicated material of adenomatous lung have also been performed and Markson and Terlecki (1964), in three separate transmission experiments, using contact, aerosols and intrapulmonary inoculation, produced jaagsiekte lesions in seven of the 22 sheep used in the study, one of which showed clinical signs 619 days after inoculation. From these results the authors concluded that SPA is a transmissible tumour.

Enchev (1966), using supernatant fluids from affected lung suspensions, reproduced pulmonary adenomatosis in five of 16 sheep. Of these five animals, clinical symptoms developed in three after nine to 23 months. The other two sheep showed no symptoms, although adenomatous lesions were found when they were killed on the 230th and 515th day respectively.

In another experiment, Enchev (1968) produced adenomatous tumour in three of 28 lambs and two of nine sheep when they were inoculated with cell-free (Seitz EK) filtrates of homogenized adenomatosis lung tissue.

Wandera (1968) reproduced the disease in two to three-week-old lambs by intratracheal or intrapulmonary inoculation with supernates of homogenised lung material

taken from sheep with histologically confirmed jaagsiekte. Of 36 animals inoculated, eight had lesions of sheep pulmonary adenomatosis, four of which showed gross lesions 100 days after inoculation. The earliest lesions were apparent 28 days post-inoculation. None of the lambs developed clinical signs and there was no variation in susceptibility with regard to age or breed. Wandera (1970b) also reported an experiment in which 10 sheep were inoculated intratracheally with sonicated tissue of tumourous lung. Lesions of SPA were found in eight sheep and, with one exception, all showed clinical symptoms.

Sharma et al (1975) described successful transmission of SPA after inoculation of lambs, between two and eight months of age, with a Seitz-filtered suspension of jaagsiekte lung tissue given by different routes. Lesions were identified in 14/180 sheep and microscopic examination revealed SPA lesions in three sheep killed six to eight weeks post inoculation. Sharma also reported that repeated intratracheal inoculation was more effective than single injections by this route or others.

Cultured cells derived from SPA tumour lesions have also been used to transmit the disease experimentally. Tustin (1969) transmitted the disease to a pair of two-day-old lambs by the intravenous inoculation of tumour cells. These cells were cultured in vitro for 10 days, during which time the culture medium had been changed twice. One lamb died of jaagsiekte after 249

days and the other showed advanced lesions in the lungs when killed after 253 days. In another experiment, conducted in the same study, unsuccessful attempts were made to induce jaagsiekte in 10 sheep inoculated intravenously with neoplastic cells obtained from another SPA tumour and grown in culture for 21 days with three changes of the medium. In another study, Tustin and Geyer (1971) conducted a similar transmission experiment but, on this occasion, tumour cells were grown for 22 days without changing the medium and the cells were inoculated into each lamb by both subcutaneous and intravenous routes. All inoculated lambs with one exception were negative for jaagsiekte. As a result of these experiments, it was considered that the causative agent of jaagsiekte might be cell-associated, since none of the lambs inoculated with medium from cultures of tumour cells showed evidence of the disease.

Coetzee et al (1976) reproduced jaagsiekte in three of eight neonatal lambs inoculated intratracheally with tumour cell culture following immunosuppresive treatment. In one lamb, the incubation period was reduced to 10 weeks. In this report the authors suggested that the viable cells present in the exhaled fluid of affected sheep represented a major source of airborne transmission of jaagsiekte since they identified a male karyotype in cells derived from tumours of female origin. Subsequently, the disease was

transmitted experimentally to neonatal lambs after intratracheal inoculation of cell-free extracts of these cells.

More recently Verwoerd et al (1980b) reported that SPA was also transmitted to newborn lambs by inoculation of a microsomal fraction of cytoplasmic extracts of cultured tumour cells or tumour tissue.

Generally, jaagsiekte has a long incubation period and clinical manifestations are observed first between two to four years of age. However, this long incubation period was reduced to as short as six months when an appropriate route of inoculation and young lambs were chosen for transmission (Wandera, 1970b).

Nevertheless Verwoerd et al (1980b) and Sharp et al (1983) have introduced an important step foward in studying SPA by dramatically reducing the lag time required to induce the disease to a few weeks by inoculating partially purified or concentrated lung fluids intratracheally in newly born lambs. These studies have shown the high susceptibility of newborn lambs to SPA, and the clinical, histological and ultrastructural similarity of the experimentally induced disease to that of natural SPA.

B). Aetiology

A great number of investigations have focussed on elucidating the aetiology of pulmonary adenomatosis and most early attempts were directed towards microorganisms such as protozoa, bacteria and nematodes as possible causes of jaagsiekte.

In 1904 Robertson described crescent-shaped bodies resembling protozoa in SPA lesions. These he thought might be the cause of the lesions, but he did not comment on whether they had any aetiological connection with the disease. However, Mitchell (1915), in a more extensive study, did not find such parasites in any of the SPA lesions or pulmonary exudates examined. He laid aside the parasite theory in favour of specific virus involvment.

Whereas McFadyean (1920) suggested a causal relationship between parasites and jaagsiekte, Dungal et al (1938) discounted this assumption on the grounds that the disease described by McFadyean was a kind of verminous pneumonia.

Claims for the isolation of a virus in chick embryo or cell culture systems from cases of SPA have also been reported (Shirlaw, 1959; Todorov and Enchev, 1966) but these reports must be viewed with caution for two reasons. First, Shirlaw isolated his virus from "Laikipia lung disease" identified by him mistakenly as jaagsiekte (Markson and Terlecki, 1964) but which was actually a complex of respiratory infections. Secondly, repeated

attempts to isolate a virus with a cytopathic effect as claimed by Todorov and Enchev were unsuccessful (Tustin, 1969).

Mycoplasmas have also been suggested as a cause of pulmonary adenomtosis (Mackay et al, 1963; Markson and Terlecki, 1964). However this suggestion was quickly discounted when it was found that the mycoplasma isolated from SPA tumours, healthy sheep, and from sheep with pasteurella pneumonia or maedi were the same (Mackay and Nisbet, 1966; Wandera, 1968; 1970a; 1971). In addition mycoplasmas were not always found in cases of SPA (Wandera, 1971). Moreover, mycoplasmas are recognized to be common inhabitants of the sheep respiratory tract (Jones, 1978).

Chlamydiae have also been isolated from the lungs of sheep with jaagsiekte (Wandera, 1971), but it is unlikely that they play a role in the aetiology of pulmonary adenomatosis in sheep since they are frequently present in apparently healthy sheep in which no SPA lesions can be detected (Page, 1968).

Two viruses have been associated with the disease, a herpesvirus and a retrovirus. All ovine herpesviruses are related antigenically and have been isolated only from SPA tumour tissues. They do not appear to cause the tumour and their association with SPA appears to arise from reactivation of latent virus in the respiratory tract (Scott, 1984).

Virus particles morphologically characteristic of RNA tumour viruses were found in thin sections of SPA tumours (Perk et al, 1971). Moreover, intracytoplasmic type A particles were described in the epithelial cells, together with type C particles budding from the plasma membrane of stromal cells in the tumour tissue. Although these particles were of unknown aetiological significance, their detection represented the first evidence for implication of a retrovirus in pulmonary adenomatosis. This observation was reinforced further by biochemical and morphological studies of purified extracts from lung tumours of sheep (Perk et al, 1974; Martin et al, 1976). These latter studies revealed viral particles with a density of 1.15-1.20 gm/ml that contain a reverse transcriptase in association with 60-70S RNA, all characteristics of retroviruses. It is significant that these particles were not seen in lung tissues of healthy sheep. Subsequently, Verwoerd et al (1980b) and Herring et al (1983) confirmed the original observations and further characterised the reverse transcriptase activity in SPA tumour tissue extracts and lung fluids. Jaagsiekte was transmitted to lambs which were inoculated intratracheally with reverse transcriptase containing fractions of tumour tissue or lung fluid (Martin et al, 1976; Verwoerd et al, 1980b; Herring et al, 1983). These experiments provided compelling evidence from the morphological, biochemical and transmission experiments that the causative agent of SPA is a retrovirus.

Sharp and Herring (1983) demonstrated a serological cross-reaction between a retrovirus structural protein (P25) contained in jaagsiekte lung material and the P27 major core polypeptide of both Mason-Pfizer monkey virus (MPMV) and mouse mammary tumour virus (MMTV), the prototypes of type D and type B retroviruses. In a study conducted at the Moredun Research Institute, a similar antigen was demonstrated in retrovirus prepared from lung fluids from SPA in several countries (Perk et al, 1985). It was concluded therefore that jaagsiekte retrovirus is serologically related, if not identical, in these countries. The findings of these studies were subsequently confirmed by Payne et al (1986) when they detected Mason-Pfizer monkey virus related antigen in the epithelial cells of SPA tumour grown in vitro, or tumour sections, by applying the immunoperoxidase test for identification. Most of the antigen was confined to the alveolar lumena of the lesion. The positive results of these histochemical studies were confirmed by immunoblotting experiments.

A number of workers have demonstrated virus particles by means of electron microscopy in the experimentally induced SPA tumour (Sharp et al, 1983; Payne et al, 1983). They described virus particles recognizable in the cytoplasm of the epithelial cells, mostly located in the vicinity of lumenal margins. These particles, measuring 60-74 nm in diameter, consisted of an electron-lucent centre encircled by two shells, the

inner shell of which was more electron-dense than the outer shell. Extracellular particles, 90-121 nm diameter, were seen only in association with the epithelial tumour cells. The particle membrane was covered with knobbed projections and encompassed an eccentric electron dense nucleoid. The description of these particles accords with that of type D and type B retroviruses. However, C type particles have never been detected in any of the tumour material. Based on morphological characteristics of the viral particles, complemented with biochemical findings, it was concluded that SPA virus is similar to type B and type D retroviruses (Sharp et al, 1983).

Attempts to isolate the SPA retrovirus from the tumour and propagate it in vitro have been disappointing. However, reverse transcriptase containing particles were demonstrated for up to four days in medium from cultured lung tumour cells (Sharp et al, 1983) and, more recently, Sharp et al (1985) made the interesting observation that the presence of SPA P25 could be demonstrated by immunoblotting for up to 96 days in the supernatant fluids of cultures from tumours induced in neonatal lambs.

CONCLUDING STATEMENT AND AIMS OF PROJECT

Considerable advances in our knowledge of the biology of human and animal neoplasms have occurred over the past years. Much of this knowledge has been obtained from studies on cell lines established from the tumour tissues. Thus, the development of continuous cultures from SPA tumours of epithelial cells uncontaminated by different cell types, is of considerable value towards our understanding of SPA tumours.

To date, there have been no successful attempts to culture SPA tumours for prolonged periods, and no detailed studies have been performed with cultures of this tumour. One report has dealt with the establishment of monolayer cultures of epithelial cells from SPA tumours (Coetzee *et al*, 1976). Unfortunately these cells have proven rather disappointing in long-term culture, and lack of identification of their cellular origin prevent true evaluation of their potential value. Therefore, the present study was undertaken with several objectives in mind:

1. Develop a technique for the isolation of epithelial cells from SPA tumours.

2. Establish cell lines from SPA tumours which retain characteristics of SPA tumour cells *in vivo*.

3. Investigate the value of these cell lines in further studies on the biology of SPA.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Medium

A single culture medium was used throughout the study as the standard medium for primary cultures, as well as for growth and maintenance of the established cell lines.

The medium used was Ham's F12K (Kaighn, 1973) with L-glutamine (KC Biological, INC., U.S.A.), supplemented with 10% inactivated fetal bovine serum (Gibco), 100 units per ml penicillin sodium salt, 100 µg per ml streptomycin sulphate, and 2µg per ml amphotericin B (Fungizone). The medium was further supplemented with 10 µg per ml insulin (Sigma Chemical Company) when used for initiation and the first few early passages of the cultures.

For maintenance of cells, the medium contained the same constituents as the growth medium, except that the concentration of fetal bovine serum was reduced to 5%.

The formulae for the medium and supplements are given in appendix 1.

Histochemical staining

Type II pneumocytes were identified by staining with Phosphine 3R (Popper, 1944) (obtained from Chroma-Gesellschaft Schmid GMBH & Co., Stuttgart, Fed. Rep. Germany) which specifically binds to the apical

cytosomes using the method described by Pfleger (1977). Three volumes of cells were suspended in medium with one volume of 0.1% Phosphine 3R in PBS (w/v). After 2-3 minutes, the cells were wet-mounted on a glass slide, viewed by fluorescence microscopy and the type II pneumocytes were enumerated. After counting 200 cells, the percentage of type II pneumocytes in the preparation was calculated.

Trypan blue staining

For assessing the viabilty of cells in suspension, the trypan blue exclusion test was used. Phosphate buffer saline containing 0.1% trypan blue was mixed with 0.9 ml of cell suspension. Viable cells, which excluded trypan blue stain, were enumerated by light microscopy using the Neubauer counting chamber. Viability of the cell suspension was expressed as a percentage of the total cell numbers.

Giemsa's stain

Monolayers of cells grown in dishes or plates were stained by decanting the supernate and fixing the cells for 10 minutes in methyl alcohol. A 1:25 solution of Giemsa's stain (Koch-Light Laboratories Ltd, Colnbrook, Berks, England), was used to stain the cells for 10 minutes then rinsed off with tap water and allowed to dry in air.

Histopathology

Tissues were fixed in Baker's calcium-formol solution and processed to paraffin wax. Sections, 5μm thick, were routinely stained with Mayer's haematoxylin and eosin.

Growth in soft agar

Monolayers of cells in the logarithmic phase of growth were trypsinised and washed twice in growth medium. Viable cells in the single-cell suspension were then counted by the trypan blue exclusion test. The suspension was adjusted to contain 10^5 viable cells per ml and then rapidly mixed with an equal volume of Ham's F12K medium containing 0.3% Noble agar and 20% fetal bovine serum (precooled to 44°C). Two-ml volumes of the resultant cell suspension in agar containing 10^5 viable cells were pipetted on to a pre-set lower layer of 0.6% agar in the same Ham's F12K medium and permitted to solidify at room temperature for 45 minutes. Before incubation, the dishes were examined for clumping of the cells and were then kept in a humidified incubator either with 5% carbon dioxide in air or a gas mixture of 5% carbon dioxide, 5% oxygen and 90% nitrogen at 37°C. The medium of soft agar cultures was replenished at 4 days intervals by adding 0.5 ml of medium containing agar. After two to five weeks, unless otherwise stated, the number of colonies with a size of more than 50 μm, were counted under a low power inverted microscope. and expressed as the mean number of three or four plates.

Chromosome analysis

Cells were seeded in 75 cm^2 plastic flasks at a density of 10^2 cells/ cm^2 and incubated at 37°C for 24-48 hours. Colcemid solution was added to the growth medium at a final concentration of 0.05 µg/ml. After 5 hours of incubation at 37°C, cells were detached from the flask surface with a solution of 0.05% trypsin and 0.16% versene, and then collected by centrifugation at 160 xg for 5 minutes. The cell pellet was resuspended in 8 ml of 0.075M KCl and incubated for 15-45 minutes at 37°C before centrifugation, as before. The cell pellet was fixed twice in methanol/glacial acetic acid, 3:1 (v/v) at room temperature for 15 and 5 minutes respectively and slides prepared by allowing one drop of fixed cells to drop from a height of about 15 cm on to clean glass slides humidified by blowing and then allowed to dry for at least 15 minutes. If the spreads were not satisfactory, the cells remaining in the tube were resuspended in 5 ml of fresh fixative, and the final incubation and centrifugation were repeated. Giemsa banding was carried out according to the method described by Seabright (1973) two days after preparing the slides. Random metaphases of well-spread chromosomes were selected for analysis by scanning the cells at low magnification and by examining them with x 100 oil immersion objective. Sixty-six metaphases were photographed. Karyotypes were prepared from photographed prints.

Electron Microscopy

A). Transmission electron microscopy

Tumour tissues were diced into $1mm^3$ blocks, immersed in 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 for two hours at room temperature, and washed three times in 0.1M phosphate buffer (pH 7.2) containing 2% dextrose. After postfixation in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.2) for 1 hour at room temperature, the tissues were washed as before, dehydrated in graded ethanols and embedded in araldite.

Ultrathin sections were cut on a Reichert Ultracut microtome and were stained with uranyl acetate and lead citrate. The sections were examined on a Siemen's IA electron microscope.

To examine cell identity and integrity in culture, confluent monolayers grown in 75 cm^2 plastic flasks were washed with PBS and fixed in 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, for 10 minutes at room temperature. The cells were then scraped off with a "rubber policeman" and pelleted at 160 xg for 5 minutes. Fixative was decanted and fresh glutaraldehyde in phosphate buffer was added and processed as described.

B). Scanning electron microscopy

Tissues were rinsed with PBS and fixed in 3% glutaraldehyde in 0.1M phosphate buffer, (pH 7.2) for 2 hours at room temperature. Samples were further processed using the modified thiocarbohydrazide procedure described by Malick and Wilson (1975).

Immunoblotting

Source of samples

Lung fluid was collected from lambs in which jaagsiekte had been induced experimentally by SPA cell lines or harvests of culture fluid collected at intervals from SPA cell cultures obtained from natural or experimental cases.

Sample preparation

Lung fluids were clarified by filtration through muslin to remove debris. Further purification, ultracentrifugation and pellet resuspension were performed as described by Sharp and Herring (1983). Pellets from culture fluids were prepared by the same procedure with the exception that the glycerol cushion was omitted.

Electrophoresis

Aliquots of samples were mixed with Laemmli sample buffer and heated in boiling water for 90 seconds, transferred to wells in a 10% polyacrylamide gel with a 3% stacking gel and subjected to gel electrophoresis for 2.5 hours at a constant current of 20 mA/cm^2 according to the method of Laemmli (1970).

Electrophoretic transfer

Electrophoretic transfer on to a nitrocellulose sheet (Schleicher and Schull; BA83) was conducted according to the procedure described by Burnette (1981) using a blotting apparatus (Electroblot) with a voltage gradient of 6.6 V/cm.

Antibody binding

Detection of the immobilized polypeptides was similar to that described by Sharp and Herring (1983).

Autogradiography was performed with X-Omat S film (Kodak) and Cronex lightning-Plus intensifying screens (DuPont).

Subculture of monolayers

Cells were routinely passed by removing spent medium and washing the monolayers twice in phosphate buffered saline, prewarmed to 37°C. The monolayers were covered with 10 ml of 0.1% Versene solution for 30 seconds, which then was decanted, and the monolayers overlaid with a further 1 ml of 0.05% trypsin and 0.16% versene solution (w/v). The flasks were rotated by hand to achieve an even distribution of the solution and incubated for 10 minutes at 37°C. During this time, the flasks were rocked every 3-4 minutes to enhance cell detachment. In most instances, the cells were detached within 10 minutes of incubation. If they did not, the flask was re-incubated for a further 5 minutes. The cells were harvested by repeated gentle pipetting of 5 ml of growth medium over the monolayer until almost all cells were in suspension. Initially 1:2 splits were made.

Preservation of cells

Cells of primary cultures and the first 5 passages of each of the four SPA derived cell lines were preserved by freezing for use as stock.

As soon as the cultures had grown to confluence in 75 cm^2 plastic flasks, the cells were harvested by trypsinizing the monolayer and suspending the cells in 20 ml of growth medium containing fetal bovine serum. The cell suspension was then centrifuged at 160 xg for 5 minutes. The medium was aspirated and the pellet resuspended in complete growth medium containing 20% fetal bovine serum and 10% dimethyl sulphoxide (DMSO). Thereafter, the cell suspension was distributed in 1.6 ml aliquots in Nunc Cryotubes and tightly stoppered. Slow freezing of the cells was ensured by placing the tubes in a closed polystyrene container after wrapping the tubes with cotton wool. Tubes were placed at -70°C overnight before transferring them to liquid nitrogen.

For recovery, the frozen cells were rapidly thawed in a 37°C water-bath. The cell suspension was immediately aspirated and suspended in growth medium, and the medium changed after overnight incubation at 37°C.

CHAPTER 3

ISOLATION AND EARLY ESTABLISHMENT OF EPITHELIAL CELLS FROM SPA TUMOURS

INTRODUCTION

SPA is considered to be caused by a retrovirus which has biochemical, morphologic and antigenic similarities to B-type and D-type retroviruses (Perk et al, 1971; Herring et al, 1983; Sharp et al, 1983). Although viral particles have been seen in the tumour cells in vivo (Perk et al, 1971; Sharp et al, 1983), growth of virus in culture has not been successful. However, cell lines derived from human teratocarcinoma and embryonal carcinomas have been reported to produce retroviruses spontaneously or after induction (Kurth et al, 1980; Bronson et al, 1984; Lower et al, 1984) and, for this reason, it was decided to initiate permanent cell lines from SPA tumour tissue in an attempt to obtain replication of the causal retrovirus. In addition, such cell lines would provide a stable population of cells permitting reproducibility of experiments and a constant background for studying cell characteristics.

Cell cultures have been initiated previously from SPA tumours (Coetzee et al, 1976), but the failure to establish these as permanent cell lines has limited

their usefulness. Furthermore, little information is available on the reproducibility of this procedure or on the yield of cells having characteristics of tumour cells. There was, therefore, a need to develop techniques for the isolation of SPA cells, and to develop permanent cell lines which retained the characteristics of SPA tumour cells.

The lung contains more than 40 different cell types (Sorokin, 1967). This extreme cellular heterogeneity of the lung makes the separation of an individual pulmonary cell type a difficult problem because this heterogeneity persists even after the pulmonary tissue is enzymatically dissociated into single cells (Gould et al, 1972).

It is generally found that attempts to isolate and cultivate epithelial cells from solid tumours of human and animal tissues have been rendered difficult for two reasons. The first is the dominance of fibroblasts in culture (Owens and Hackett, 1972; Owens et al, 1976), and the consequent loss of epithelial cells. This dominance arises from the ability of fibroblasts to adhere to the surface of culture vessels faster than epithelial cells (Lasfargues, 1973), coupled with a shorter generation time in vitro (Douglas and Kaighn, 1974). Secondly, the macrophages that are frequently present in solid tumours (Evans, 1972), if not removed, can be activated in vitro and kill the tumour cells (Evans, 1972; Heskill et al, 1975; Currie, 1976).

In the present attempts to isolate SPA tumour cells it was considered that a procedure which overcame these two problems would be desirable.

The proliferation of fibroblasts may be controlled at two stages; either by their removal during the dissociation and isolation procedure (Williams et al, 1971; Lasfargues, 1973; Coetzee et al, 1976) or by inhibition of their growth in vitro by such means as selective trypsinization (Owens et al, 1974; Lasfargues, 1973), selective media (Gilbert and Migeon, 1975; White et al, 1978; Vesterinen et al, 1980; Pathak et al,1982), chemical treatment (Kao and Prockop, 1977; Penter et al, 1978), exploitation of functional differences (Lubaroff, 1977; Jensen and Therkelsen, 1981; Gusterson et al, 1981; Namba et al, 1983; Watanabe et al, 1983; Baillie-Johnson et al, 1985), or clonal culture (Coon, 1966; Epstein and Fukuyama, 1973; Douglas and Kaighn, 1974; Stoner et al, 1975; Kniazeff et al, 1976; Douglas and Farrell, 1976; Giovanella et al, 1976; Ishiwata et al, 1977; Tanswell and Smith, 1979; Paraskeva and Gallimore, 1980).

Another major problem in culturing epithelial cells from solid tumours is the existence of macrophages (vide supra). The removal of macrophages from mixed cell populations has been achieved by exploiting the functional and physical properties of this cell type such as phagocytosis, density range or cell size (Kikkawa and Yoneda, 1974; Greenleaf et al, 1979; Mason et al, 1975; Pfleger, 1977; Dobbs et al, 1980; Lafranconi et al, 1983), or

differential adhesion in culture (Fisher et al, 1980; Mettler et al, 1981; Goodman and Crandall, 1982). Contaminating macrophages and lymphocytes have been eliminated also by specific surface adsorption, exploiting the fact that these cells express leukocyte common antigen (LC) on their surface, whereas other cells do not (Weller and Karnovsky, 1986a & 1986b).

Unfortunately, the available literature does not identify a single best approach and for this reason a number of procedures were evaluated in the preliminary studies.

MATERIALS AND METHODS

Source of tumour

Sheep that showed clinical signs of SPA, were obtained from flocks throughout Scotland. They were killed by intravenous injection of pentobarbitone and the lungs removed from the thorax. Pieces of tumour were taken for histopathological examination to confirm the clinical diagnosis.

Medium and solutions

Culture medium

The growth medium used was Ham's F-12K supplemented with 10% fetal bovine serum as described in General Materials and Methods. In this study crystalline bovine insulin (Sigma), dissolved in 0.1N hydrochloric acid, was incorporated in the growth medium at a final concentration of 20 µg/ml.

Trypsin

The dissociation mixture consisted of 0.25% trypsin (Difco) in Tris buffer, pH 7.4 (Appendix 1) containing 1% chicken serum and antibiotics (penicillin, 200 I.U.; streptomycin, 200 mg/ml and polymyxin B, 5 µg/ml). All these supplements were added immediately prior to use.

Cell Culture Procedure

Part 1

Preliminary evaluation of procedures to inhibit or control fibroblast proliferation

A). Selective and prolonged trypsinization procedure

The procedure described by Coetzee et al (1976) employed prolonged trypsinization for disaggregation of tumour followed by selective trypsinization of cultures to eliminate contaminating fibroblasts. Continuous supplementation of the medium with betamethasone further depressed the growth of any remaining fibroblasts while promoting that of epithelial cells. This procedure was used in the present study.

B). Growth of cells in medium with D-valine

MEM medium in which D-valine was substituted for L-valine, was used as a simple method to selectively inhibit fibroblast growth (Gilbert and Migeon, 1975). The selectivity of this medium is based on the fact that the enzyme D-amino acid oxidase which is present only in specialized epithelial cells, is absent in fibroblasts. Growth of fibroblasts is inhibited because they cannot connvert the essential amino-acid valine from the D to L form. In the present study, this medium, supplemented with 10% dialyzed fetal bovine serum, was used to grow SPA epithelial cells.

C). Iodoacetic acid sodium salt

The sodium salt of iodoacetic acid was added to SPA cells at a concentration of 1.5×10^{-5} M as recommended (Penter et al, 1978). The inclusion in the medium of this chemical was attempted using both primary cells and subcultures.

D). Medium supplemented with allo-hydroxyproline

The selective effect of allo-hydroxyproline was investigated by continuously maintaining SPA cultures in medium containing 100 µg/ml of this chemical. Cells were replenished with medium containing allo-hydroxy-proline every 48 hours and passaged six times, during which time the growth of fibroblasts was monitored.

E). Cloning in soft agar

To determine whether this would be a useful technique for the isolation of malignant cells from SPA tumours, the isolated cells were cloned directly into soft agar as stated in "General Materials and Methods"

Dissociation procedures

Pieces selected from SPA tumours were cut into small fragments, dissected free of connective tissue, then washed in several changes of PBS. The tumour fragments were placed in a sterile plastic petri dish and chopped finely with curved scissors. The minced tissues were then transferred to a beaker and washed at least

three times in PBS to remove debris and blood clots. With each wash, the fragments of tissue were allowed to settle before the supernate was removed. From this point, the minced tumours were disaggregated to yield a single cell suspension either by trypsinization for 48 hours (Coetzee et al, 1976) or by three 15 minute cycles of trypsinization. The two procedures are described below.

Part 2

Evaluation of the adopted procedure

Long trypsinization

For separation of cells, the washed mince was placed in a 2 litre Ehrlenmeyer trypsinizing flask containing 600 ml of cold 0.25% trypsin solution at pH 7.4. The trypsin solution was supplemented with 1% chicken serum to maintain cell viability during this long procedure (Douglas et al, 1976). The tissue mince in trypsin solution was slowly agitated with a teflon--coated magnetic bar at a rate sufficient to keep the fragments in gentle motion for 48 hours at 4°C. At the end of this period, the action of the trypsin was prevented by adding fetal bovine serum to a concentration of 2%, whereupon undigested fragments were removed by filtration through a double layer of surgical gauze. The resulting cell suspension was then poured into sterile 30 ml plastic tubes (Sterilin) and centrifuged for 5 minutes at 160 xg at 4°C (TH-4 Rotor, Beckman TJ-6R Centrifuge).

The medium was then decanted and the pellets resuspended in fresh medium. The cell suspension was washed 2-3 times in this manner.

At every step, the total cell count was determined using a haemocytometer, the cell viability by Trypan blue exclusion and the proportion of type II pneumocytes by staining with Phosphine 3R as described in " General Materials and Methods"

Short trypsinization procedure

The washed mince was transferred to a 250 ml capacity Ehrlenmeyer flask containing 100 ml trypsin solution previously warmed to 37°C. The suspension was agitated gently with a magnetic bar at 37°C. After 5 minutes, the stirring was stopped, the undigested fragments allowed to settle, and the supernate from this trypsinization discarded. Fresh trypsin was added and the tissue was incubated with stirring for 15 minutes. After this time, the dissociated cells were harvested and serum was added at 2% to prevent further trypsinization. The harvested cells were kept at 4°C whilst two further 15 minute cycles of trypsinization were performed. The pool of harvested cells was dealt with as described in the 48 hours trypsinization procedure.

Cell separation

Removal of phagocytic cells

Following trypsin dissociation, phagocytic cells were removed by the carbonyl-iron technique with some modification (Buick and Salmon, 1980). Before use, the carbonyl-iron powder was washed three times in PBS and once in medium to reduce toxicity. A suspension of 10^8 cells in 20 ml of medium containing 1% fetal bovine serum and 10 mg/ml carbonyl-iron was incubated and slowly rotated for one hour at 37°C. Cells that had ingested carbonyl-iron particles together with the free carbonyl-iron particles were depleted from the suspension by lateral attraction to a magnet and the remaining non-phagocytic cells were collected. This procedure was repeated until it was considered by visual inspection that all the carbonyl-iron had been removed.

Removal of fibroblasts

The other step in the isolation procedure was differential adherence (Mason *et al*, 1976) which was employed to selectively remove epithelial cells from the more rapidly adherent cells, consisting mainly of fibroblasts and residual macrophages. After carbonyl-iron treatment, the non-phagocytic cells were washed twice in growth medium and then plated in plastic flasks at 10^6 cells/cm^2 and incubated for three hours at 37°C in a defined gas mixture (5% CO_2, 5% O_2, 90% N_2). The non-adherent cells were aspirated and reseeded in plastic flasks.

Culture of cells

The remaining nonphagocytic, slowly adherent cells were suspended in culture medium at a concentration of 2×10^5 cell/cm² (30 ml/ 75 cm² flasks). The cultures were gassed as above for a few seconds then tightly closed and allowed to attach overnight at 37°C. The next day, culture medium containing the remaining cells was discarded and the attached cells were replenished every other day through day 7 to ensure suffient nutrients for growth. Cultures were maintained in the same gas mixture after each change of medium.

RESULTS

Preliminary evaluation of procedures to inhibit or control fibroblast proliferation

A). Selective and prolonged trypsinization procedure

The procedure attempted in the present study was that described by Coetzee et al (1976). Spindle-shaped cells were seen in culture among the predominating epithelial cells. Betamethasone treatment not only stimulated cell proliferation as evidenced by excessive piling up of small epithelial cells in culture but also enhanced the appearance of large cells. These large cells were firmly attached to the flask surface and were less sensitive to the effect of trypsin-versene as a long time was required to detach the cells with this mixture. Furthermore, the use of betamethasone did not deter fibroblast growth and attempts to separate the fibroblasts from the epithelial cells by selective trypsinization were unsuccessful. The spindle cells and small actively proliferating epithelial cells became detached somewhat equally and more rapidly, while the other cell types present in the culture remained attached to the surface. Attempts to remove fibroblasts by selective trypsinization resulted in monolayers of large slow growing cells but such cultures did not grow beyond the 10th passage.

B). Growth of cells in medium with D-valine

Cultures of SPA tumour cells grown in D-valine medium showed drastically reduced numbers of fibroblastic cells, and most cells were epithelial in appearance. However, after 6 passages, fibroblasts could still be detected. In addition, the growth of the predominant epithelial cells was impaired as reflected by slow growth, abnormal appearance, vacuolation and cell death. Eventually, by the 8th passage there was no cell division and the cultures were lost.

C). Medium supplemented with iodoacetic acid

When iodoacetic acid sodium salt was added to the culture medium, as indicated in "Materials and Methods", both fibroblasts and epithelial cells were killed in less than 12 hours of incubation. Cells exposed to this chemical showed flattened morphology, disintegrating nuclei and became detached into the culture medium.

D). Medium supplemented with allo-hydroxyproline

Allo-hydroxyproline, added to the medium as recommended by Kao and Prockop (1977), did not deter the proliferation of fibroblasts in SPA cultures. Indeed, fibroblasts dominated the culture, despite being maintained in the presence of allo-hydroxyproline for six consecutive passes.

E). Soft agar

In soft agar none of the cultures demonstrated the capacity to grow despite the use of supplements to enhance growth as shown in Chapter 4.

Evaluation of preliminary experiments

The aim of this study was to develop a routine procedure for the initiation of pure epithelial cells by creating growth conditions that support epithelial proliferation but suppress or inhibit the growth of fibroblasts. The methods adopted by previous workers (Gilbert and Migeon, 1975; Coetzee et al, 1976; Kao and Prockop, 1977; Penter et al, 1978) were not found to be suitable for SPA cultures, either because fibroblasts were not completely eliminated from the culture or the growth of epithelial cells was not maintained.

During the preliminary experiments it was observed that cultures derived from SPA tumours contained large number of macrophages which adhered rapidly and covered the surface of the flask thereby interfering with the attachment of epithelial cells. Thus it became clear that in addition to proliferation of fibroblastic cells, the presence of phagocytic cells presented a further obstacle to obtaining culture of epithelial cells from SPA tumours. For this reason attention was diverted towards developing a procedure which would overcome these obstacles.

In doing so it was decided to introduce and assess a procedure which exploited some simple functional properties of the various cell types. For example, fibroblastic cells are known to be more sensitive to trypsin, and adhere faster than epithelial cells. Also, macrophages have the ability to phagocytose particulate matter. Thus, a combined procedure was devised in which the tumour was disaggregated by prolonged trypsinization to kill fibroblastic cells. Macrophages were depleted from the resulting cell suspension by ingestion of carbonyl-iron particles (magnetism) followed by a period of adherence to separate epithelial and lymphocytic cells from non-epithelial cells.

Part 2

Evaluation of the adopted procedure

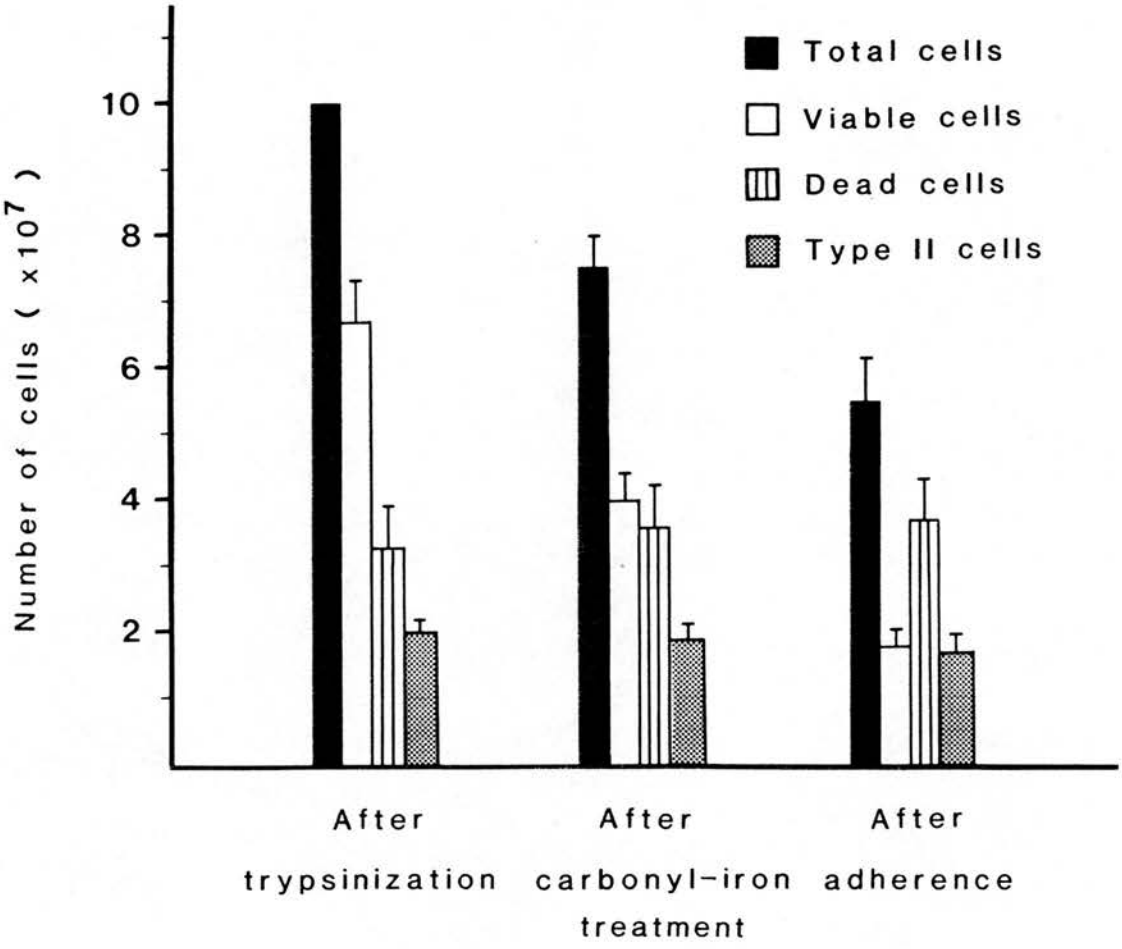
At the outset of this procedure, short and prolonged trypsinization of tumour tissues were used to determine differences in viability and to find if there were any differences in concentration of type II pneumocytes or in purity of the suspensions.

Long trypsinization

Results presented in Fig. 3.1 were obtained from six different tumours. In a population of 10^8 of unfractionated SPA cell suspension, the average yield of viable cells after trypsinization was 67.2×10^6, of which 20.3×10^6 (30.2%) cells stained positively with Phosphine 3R.

Fig. 3.1

Effect of the isolation procedure on the viability and yield of type II pneumocytes from SPA tumours treated for 48 hours with trypsin.


Total cells
Viable cells
Dead cells
Type II cells
Number of cells (x10^7)
10
8
6
4
2
After trypsinization
After carbonyl-iron treatment
After adherence

Following carbonyl-iron treatment of the cell suspension, an average total of 27.4 x 10^6 (40.8%) cells was removed, leaving 39.8 x 10^6 viable cells. Of these remaining viable cells the average number staining with Phosphine 3R was 19.3 x 10^6 representing 48.5%.

The adherence procedure further reduced the viable cells by 22.1 x 10^6 cells indicating that 55.5% were rapidly adherent cells such as fibroblasts and macrophages. The remaining 17.7 x 10^6 viable cells contained 17.1 x 10^6 (96.6%) cells staining with Phosphine 3R which represents a loss during the three steps of only 3.2 x 10^6 (15.8%) of this cell type.

Short trypsinization

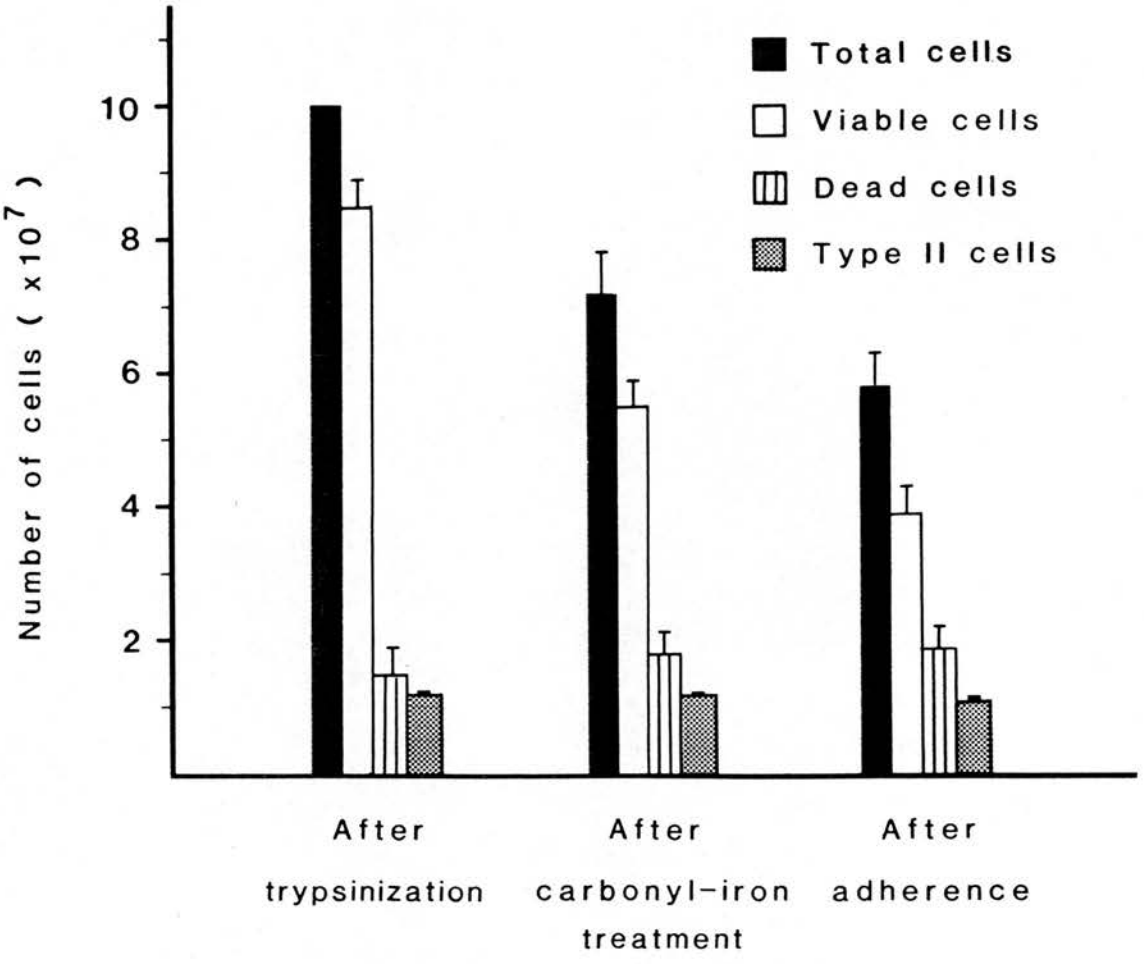
The results from disaggregation of 5 different tumours are presented in Fig. 3.2. In a population of 10^8 cells the average yield of viable cells was 85 x 10^6 cells in which 12.4 x 10^6 (14.6 %) cells stained positively with Phosphine 3R.

After the cell mixture was treated with carbonyl-iron an average total of 30.6 x 10^6 cells were removed. This figure shows that 36% of viable cells obtained by disaggregation were phagocytic. In this cell mixture the average yield of type II pneumocytes was 11.8 x 10^6 cells, a value representing 21.6% of the total viable cells.

The adherence procedure further reduced the viable cells by 15.4 x 10^6. This indicates that 17% of the

Fig. 3.2

Effect of the isolation procedure on the viability and yield of type II pneumocytes from SPA tumours after short treatment with trypsin.


Total cells
Viable cells
Dead cells
Type II cells
Number of cells (x10^7)
10
8
6
4
2
After trypsinization
After carbonyl-iron treatment
After adherence

viable cells were rapidly adherent cells. The remaining 39.3×10^6 viable cells contained 11×10^6 type II cells representing 28% of the remaining viable cells.

Growth and morphology of cells during early passes

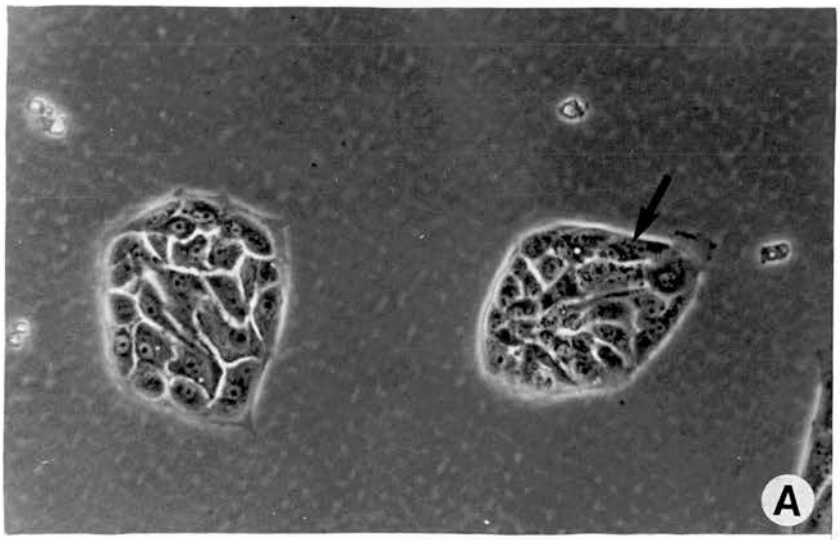
When cell suspensions obtained by the long trypsinization technique were grown in culture, monolayers of epithelial cells free from contaminating fibroblasts developed. However, plating of cells after short trypsinisation resulted in most instances in the presence of some dendritic cells among the colonies of epithelial cells and their removal required mechanical destruction.

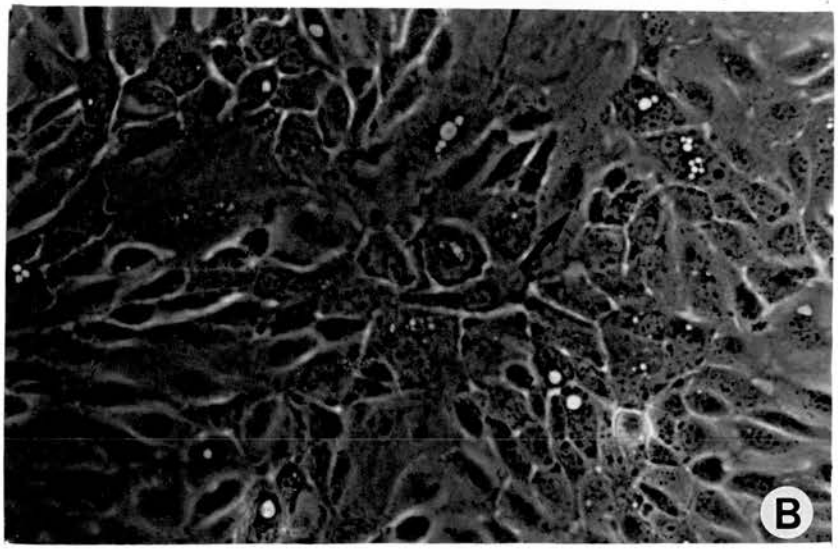
Cells obtained by the prolonged trypsinization procedure were plated at a density of 2×10^5 cells/75 cm^2 culture flasks containing 30 ml growth medium. After 24 hours, islands of cells developed which showed a somewhat polygonal morphology with a very smooth edge (Fig. 3.3A). The cells contained vacuoles of varying sizes and numerous intracytoplasmic dark granules which were generally close to the nuclear membrane and could be easily seen by light or phase contrast microscopy (Fig. 3.3B).

During the first 48 hours the cells grew rapidly and by the 3rd or 4th day the cell sheets were almost confluent. One day later two types of cells could be observed in the cultures; a small cuboidal cell and a large cuboidal cell. The small cuboidal cells, which assumed a close-packed arrangement, appeared to divide

Fig. 3.3A,B

Primary culture of SPA cells. (A), 36 hours after plating; (B), 4 days after plating. The cells show features of type II pneumocytes, such as epithelial morphology and intracytoplasmic granules (arrows). Phase contrast. x 200.


A


B

rapidly. Generally the cytoplasm of these cells was vacuolated and contained granules. The nuclei were large and occupied most of the cell. The boundaries of many cells were refractive which was especially obvious when the cultures were viewed by phase contrast microscopy.

The other type of cell was large and cuboidal (Fig.3.4). The nuclei were small in relation to the cytoplasm and most cells contained two nuclei. Many cells contained multiple vacuoles which occasionally occupied the entire cytoplasm. The cytoplasm of some cells was fragmented and sloughing of occasional cells was evident. In subsequent passages these cells showed a slow growth rate and firm attachment to the substratum.

When the cultures reached confluence between the 5th and 7th day, the small cuboidal cells tended to pile up forming multilocular or cord-like structures of rounded cells (Fig. 3.5). This piling of cells was accelerated by continuous feeding of the cultures with fresh medium. In some islands, the cells started to pile up long before the monolayers reached confluence. In due course the piled up colonies became detached from the monolayer. Cultures that were developed from the detached cells were composed of two cell types which were similar in morphology to the original cultures.

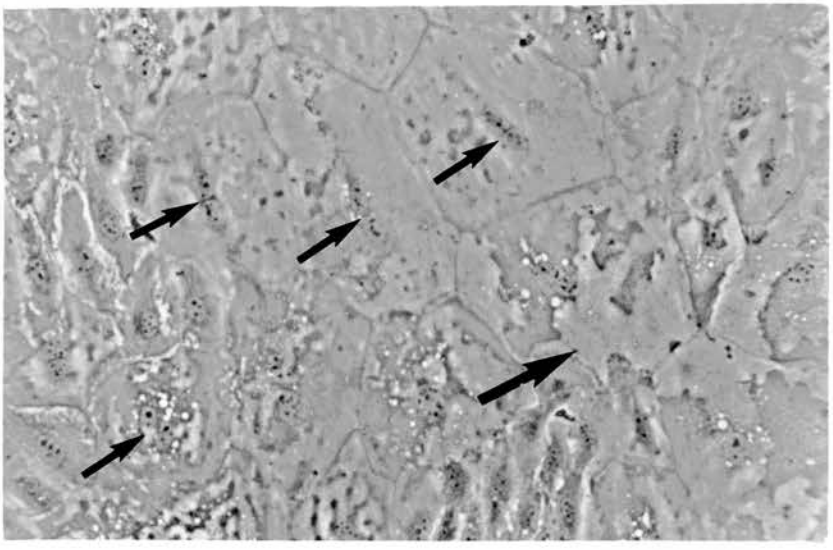
During early passages, (10 to 15 passages) the cultures were transferred every 4-5 days at a ratio of 1:2 before the piling cells started to detach. The cells

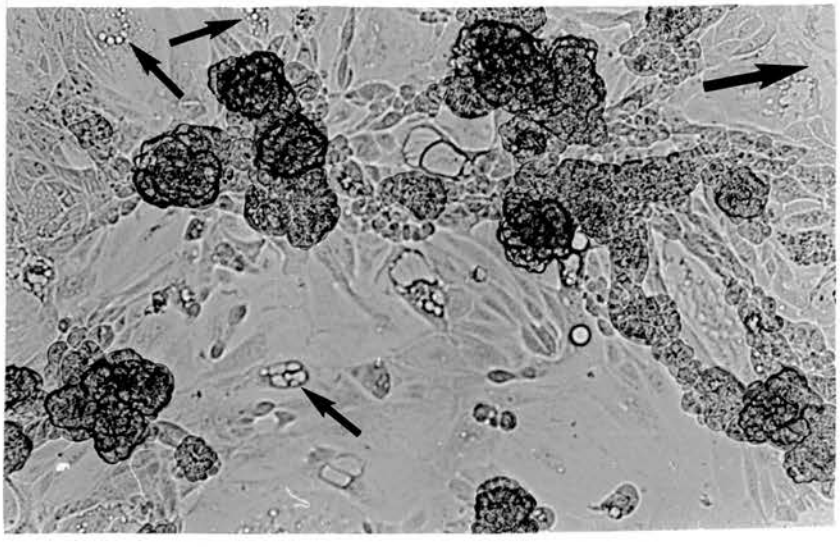
Fig. 3.4

A focus of an eight-day-old culture showing flattened large cells with clear boundaries and each cell exhibiting two nuclei (small arrows). Cell vacuolation is evident. Extensive breakdown of cytoplasm into fragments seen in some cells (large arrow). Phase contrast x 200

Fig. 3.5

A six-day-old culture. Note small epithelial cells forming piles of rounded cells arranged into rose-shaped or cord-like structures, indicating loss of contact inhibition. Large cells with vacuolation (small arrows) or fragmented cytoplasm (large arrow) are seen. Phase contrast x 200





after the 10th or 15th passages were able to form complete monolayers in 5 days when divided at a ratio of 1:4 . At these passage levels, the small active cells became predominant at the expense of the large cells. In some cultures these large cells completely disappeared from the cultures after 20 passages.

Piling up of cells in culture was a consistent features, with the exception of JS14 where the cells showed marked contact inhibition (see Chapter 4).

DISCUSSION

Development of epithelial cell cultures from SPA tumours requires isolation procedures that preserve the isolated tumour cells from the phagocytic activity of macrophages and the competitive outgrowth of fibroblasts which grow more rapidly than epithelial cells.

The three-step procedure reported in the present study (extreme trypsinization, carbonyl-iron to remove phagocytic cells, and adhesion) was found to be efficacious for the isolation of epithelial cells from SPA tumours. It was also more satisfactory than short trypsinization with regard to the yield of type II pneumocytes and the absence of fibroblast contamination.

In the early attempts to propagate epithelial cells from SPA and to contain the proliferation of fibroblasts, several approaches were attempted (MacPherson and Montagnier, 1964; Owens et al, 1976; Kao and Prockop, 1977; Penter et al, 1978; Coetzee et al, 1976). Medium containing D-valine is said to inhibit fibroblast growth and maintain the proliferation of epithelial cells (Gilbert and Migeon, 1975; White et al, 1978; Vesterinen et al, 1980; Pathak et al, 1982). However, other reports have shown the failure of this medium to maintain the growth of epithelial cells or inhibit the proliferation of fibroblasts (Gilbert and Migeon, 1975; Dodson et al, 1978; Biran et al, 1983). This has been attributed to the absence of the necessary

enzyme D-amino acid oxidase in certain epithelial cells. In the present study, although D-valine medium limited the proliferation of fibroblasts in culture, it also failed to sustain long-term growth of the necessary epithelial tumour cells. Thus the failure of SPA epithelial cells to grow in this medium suggests that these cells are lacking the enzyme D-amino acid oxidase.

Coetzee et al (1976), using prolonged trypsinization to disaggregate the tumour, noted that primary cultures initiated from lung tissue with SPA tumour contained few fibroblast cells. These fibroblasts were selectively removed by means of repeated mild trypsinization of cultures maintained in medium whilst epithelial cells remained attached (Owens et al, 1976). In the study reported herein, epithelial cells, in particular the actively proliferating cells, appeared to be as sensitive to the trypsin-versene treatment as the fibroblasts and both cell types detached at equal rates. These observations are similar to the results obtained for the initiation of epithelial cell lines from naturally occurring ovine squamous cell carcinoma (AL-Yaman and Willenborg, 1984) and precluded use of this procedure.

Penter et al (1978) noted a selective killing of fibroblasts by iodoacetic acid, but in the present study inclusion of this chemical in SPA cultures resulted in cytotoxicity and killed all cells in a short time. Probably, this difference was due to the fact that different cell types were used or, possibly, to the toxic

effect of the iodoacetic acid as a result of employing too high a concentration of this chemical.

Allo-hydroxyproline reduced fibroblasts in SPA cultures but did not completely inhibit their purification. This observation might be explained by the findings of Kao and Prockop (1977) who reported that some populations of fibroblasts were more resistant than others and that higher concentrations of cis-hydroxyproline were needed to inhibit the growth of fibroblasts.

Identification of type II cells is based initially on their histochemical, morphological and ultrastructural properties. In this study, the type II cells were identified by their characteristic fluorescence with Phosphine 3R and the detection of refractile granules in the cytoplasm, usually in the perinuclear region. These criteria appear to be reliable markers of the transformed type II cells in SPA tumour because, in other experiments, macrophages, fibroblasts and epithelial cells obtained from normal lung did not demonstrate these features. Electron microscopy could not be used to confirm their identity because the residual iron particles remaining in the cell suspension presented a technical difficulty in cutting ultrathin sections. A similar problem was encountered by Lafranconi et al, (1983).

In both short and long trypsinization, phagocytic cells constituted 36% and 40.8%, respectively, of viable cells in the disaggregated cell suspensions.

These findings are in agreement with those of earlier workers which showed that, in cell suspensions from experimental murine tumours, up to 50% of cells were macrophages (Evans, 1972; Van Lovern and Den Otter, 1974; Wood and Gillespie, 1975; Kerbel and Pross, 1976; Holden et al, 1976; Russel et al, 1976; Wood and Gollahon, 1977). In human tumours also, a high content of macrophages infiltrating tumour tissues has been reported (Gauci, 1976; Wood and Gollahon, 1977; Harvy and Totterman, 1978). The abundance of macrophages in SPA tumour suggests that an immune response is occurring within the tumour, and the content of macrophages may affect the prognosis of the disease by controlling the spread of the tumour (Eccles and Alexander, 1974; Wood and Gillespie, 1975; Lauder et al, 1977).

Although SPA tumour cells were obtained by long and short trypsinization, 48 hours was preferred for two reasons. The initial technique used for obtaining the cell suspension, which involved prolonged trypsinization, resulted in more type II pneumocytes (30.2% vs 14.6%). These results indicate that long trypsinization had a detrimental effect on the viability of fibroblasts which are markedly reduced in the initial cell suspension. Furthermore, the cell suspension obtained from prolonged trypsinization, when placed in culture after a further purification procedure, generated cultures of epithelial cells completely free from fibroblasts. This observation is in keeping with the results of Coetzee et al (1976)

who found that fibroblasts in SPA tumour suspensions are more sensitive to trypsin and that long digestion with trypsin resulted in the removal of most fibroblasts, but without unduly affecting the epithelial cells. In contrast, although the final cell suspension originating from the short trypsinization had a higher viability, only 28% were type II alveolar cells, indicating considerable contamination by other cell types. This was further apparent in culture where fibroblasts were seen among epithelial islands.

While the viability of SPA cell suspensions obtained by short trypsinization is high, the lower proportion of type II pneumocytes coupled with the growth of fibroblasts represents distinct disadvantages.

The method finally adopted in the present study was both efficient and reproducible, and eliminated most of the fibroblasts and macrophages from the mixed cell suspension before placing it in culture. The method yielded cell suspensions enriched to 96.6% purity for epithelial type II cells from SPA tumours. These results are comparable to those obtained from rabbit lungs by Lafranconi et al (1983) with a combination of three basic techniques namely, critical enzyme placement by perfusion of collagenase and elastase through the pulmonary artery to remove non-epithelial cells, magnetic removal of macrophages, and cell sizing through sieves to remove contaminating cell types.

During the three-step procedure in the present study both the total number and proportion of viable cells were reduced markedly (Fig. 3.2), whereas the number of dead cells increased slightly. This indicates that treatment with carbonyl-iron and adherence removes only viable cells and that the reduction in viable cells is due to the removal of phagocytic cells and fibroblasts. In contrast, the total number of type II cells was reduced by only 3.2×10^6 cells and their proportion increased from 30.2% to 96.6%.

Pfleger (1977) has reported that Phosphine 3R produces characteristic fluorescence and a distinct pattern of granules only in viable type II cells. Therefore, in the present experiments the increasing proportion of type II cells appears to be due to an enrichment of viable type II cells rather than to an accumulation of dead cells, which also would not be removed by the procedures employed.

In this study, it was observed that two epithelial cell types exist in early passes of cultures initiated from SPA tumour. Others have reported two morphologic cell types in cultures from rabbit lung tissue or SPA tumours (Coetzee et al, 1976; Diglio and Kikkawa, 1977). The origin of the large cell type observed in this and other studies is unclear (Coetzee et al, 1976; Diglio and Kikkawa, 1977). It may reflect a feature of cell senescence, different stages of differentiation of the same cell or, alternatively, may represent type I cells

derived from type II cells (Diglio and Kikkawa, 1977). The latter view is supported by the observations of Adamson and Bowden (1974) that type II transformation to type I does occur in vivo.

The successful growth of epithelial cells from SPA tumour may have been the result of several factors. Thus, the removal of phagocytic cells from SPA cell suspensions before they are placed in culture may have provided an important stage for the initiation of epithelial cultures by protecting the tumour cells from the destructive action of phagocytic cells. The use of a gas phase in which the oxygen is lowered to 5% might be important in maintaining the epithelial culture. In this regard, Richter (1973) reported the superior growth of many mammalian cells in an environment low in oxygen. Courtenay (1976) showed that to obtain growth of Lewis lung tumour or the B16 mouse melanoma cells in culture from small inocula, the oxygen concentration in the medium may be critical. She suggested that the use of 5% oxygen resulted in considerable improvment in the plating efficiency of these cells in culture. Also, in the studies shown in Chapter 4, it was found that a gas phase containing 5% oxygen was necessary for the growth of SPA tumour cells in soft agar.

Another factor which may have helped in the successful growth and maintainence of epithelial cells in culture is the addition of insulin to the medium. From the literature, this hormone has been utilized successfully

either singly or in combination in many culture systems to maintain mammalian epithelial cells (Mather and Sato, 1977).

In conclusion, the studies presented in this Chapter demonstrate that it is possible to obtain epithelial cell cultures from SPA tumour by the combined technique described in the text and it is hoped that the procedure will be of value to other investigators who wish to obtain cell cultures of SPA epithelial cells.

CHAPTER 4

MORPHOLOGY AND GROWTH CHARACTERISTICS OF SPA DERIVED CELL LINES.

INTRODUCTION

In the preceding Chapter, a technique was described which facilitated the initiation from SPA tumour of several cell cultures exhibiting epithelial morphology.

The development of permanent cell lines from such material might aid in the investigation of tumourigenesis in both sheep and humans, since there are considerable histological and ultrastructural similarities between SPA tumour and human bronchioloalveolar carcinoma (Nobel and Perk, 1978).

Cell culture has long been documented as an appropriate system in which to identify intrinsic differences between normal cells and cancer cells and to determine how an alteration manifested as abnormal behaviour in culture gives rise to malignant behaviour *in vivo*.

Transformed cells exhibit a number of *in vitro* biological characteristics which distinguish them from their normal counterpart. For example, changes in cell morphology, increased saturation density, decreased doubling time, loss of contact inhibition, modified cell surface components, abnormal karyotype, anchorage-independent growth and the capability of continual

subcultivation *in vitro*. Some of these abnormal features have been found to correlate with the ability to form tumours in immunosuppressed or immunodeficient experimental animals (Sanford, 1968; Yosida, 1968; Shin *et al*, 1975, Stiles *et al*, 1976; Fogh *et al*, 1977).

In the present study, SPA cell lines have been examined for similar characteristics, specifically, growth kinetics, karyotype alterations and *in vitro* growth parameters. These features have been evaluated in an attempt to use them as a means of predicting tumourigenic potential of these SPA epithelial cells. Additionally, cells were studied for possible virus replication during culture

Four cell lines were selected from the epithelial cultures described in Chapter 3, because they exhibited different properties. Two of the cell lines, JS7 and JS15, contained a high proportion of cells that stained positively with Phosphine 3R and therefore appeared similar to type II pneumocytes. The third SPA cell line, JS8, appeared similar to JS7 and JS15 but contained fewer type II cells. The fourth cell line, JS14, contained few type II cells but showed contact-inhibited growth.

The work in this chapter is divided into 4 subsections;

A). General description of the cell lines.

B). Growth of cells in soft agar.

C). Functional characteristics.

D). Retrovirus antigen detection in SPA cells.

A). GENERAL DESCRIPTION OF CELL LINES

MATERIALS AND METHODS

Medium

The medium used was the Ham's F-12K medium as described in "General Materials and Methods".

Cells

The four different cell cultures designated JS7, JS8, JS14 and JS15 were used.

Staining methods

Giemsa's stain was used to visualize colonies developed on plastic and Phosphine 3R was used to identify type II cells. Both staining techniques are described in "General Materials and Methods".

Attachment efficiency

Attachment efficiency (Shouval *et al*, 1981; Wu *et al*, 1982) of each individual cell line was determined by seeding 2 x 10^5 viable cells in two 60 x 15 mm plastic tissue culture dishes. Twenty-four hours later, the medium was removed and the cultures washed twice with PBS to remove unattached cells. The attached cells were removed by trypsinization then harvested and counted with a haemocytometer. Attachment efficiency was expressed as the percentage of seeded cells attached within 24 hours of incubation according to the formula (number of cells attached to the surface of culture plate/ number of cells seeded in each plate x 100).

Plating efficiency on plastic

In order to determine colony forming efficiency on plastic, 2 ml of medium containing 100, 250 or 500 cells were seeded in each of four replicate wells of plastic plates (Linbro, cat. no., 76-053-05). The plates were incubated in a humidified atmosphere of 5% CO_2. The culture medium was changed every other day to ensure sufficient nutrients for cell growth. Seven days after inoculation, the cultures were washed twice with Phosphate-buffered saline, fixed in methanol and stained with 5% (v/v) Giemsa solution to show-up the colonies. One colony was defined as a population of 8 or more contiguous cells. The total number of colonies per well was obtained by direct counting using an inverted microscope at x 10 magnification.

Plating efficiency was expressed as the mean percentage of inoculated cells which formed colonies seven days after inoculation (PE = number of colonies counted in each plate/ number of plated cells x 100).

Growth kinetics

Twenty, 60 x 15 mm plastic tissue culture Petri dishes were incubated with 2 x 10^5 viable cells in 5 ml growth medium. The cells were suspended at a density of 2 x 10^4 cells/ml and during dispensing they were agitated gently with a pipette to ensure even dispersion and even distribution in each dish. Culture plates were then incubated at 37°C in a humidified atmosphere of 5%

CO_2. Cultures were fed every 24 hours by complete removal of the spent medium and replacement with 5 ml of fresh growth medium to maintain maximum growth conditions. At 24-hours intervals for 10 days, cells from two cultures were trypsinized, and cell counts determined. The doubling times were estimated in the logarithmic growth phase.

Saturation density

To determine the saturation density of individual cell lines, cultures were prepared by seeding 2×10^5 cells into 25 cm^2 Nunc plastic flasks and incubing them at 37°C in a humidified atmosphere of 5% CO_2. Cultures were fed every second day to maintain sufficient nutrients for growth. When a confluent sheet was established, duplicate cultures of each cell line were harvested with trypsin-versene every two days and enumarated with a haemocytometer. Saturation density was calculated from the point when no further increase in cell numbers per flask was observed or, as with some cell lines, when the numbers began to decrease as the cells detached from the surface of the flask. Saturation density was expressed as cells/cm^2.

Chromosome analysis

Cells were grown in 75 cm^2 plastic flasks at a density of 10^5 cells/cm^2 for 24-48 hours. Cells were incubated in medium containing Colcemid (0.05 µg/ml) for 5 hours at 37°C, harvested and processed as described in "General Materials and Methods".

Transmission electron microscopy

The method used to examine the ultrastructure of JS7, JS8, and JS14 cells was as described in " General Materials and Methods".

RESULTS

Growth properties

Cell lines grew as islands consisting of closely packed cuboidal or polygonal cells which eventually coalesced to attain confluence in 3-4 days (Fig. 4.1). With the exception of line JS14, the nuclei were large in comparison to the cytoplasm and contained more than one nucleolus. In all cell lines, cytoplasmic vacuoles and binucleate cells were observed frequently.

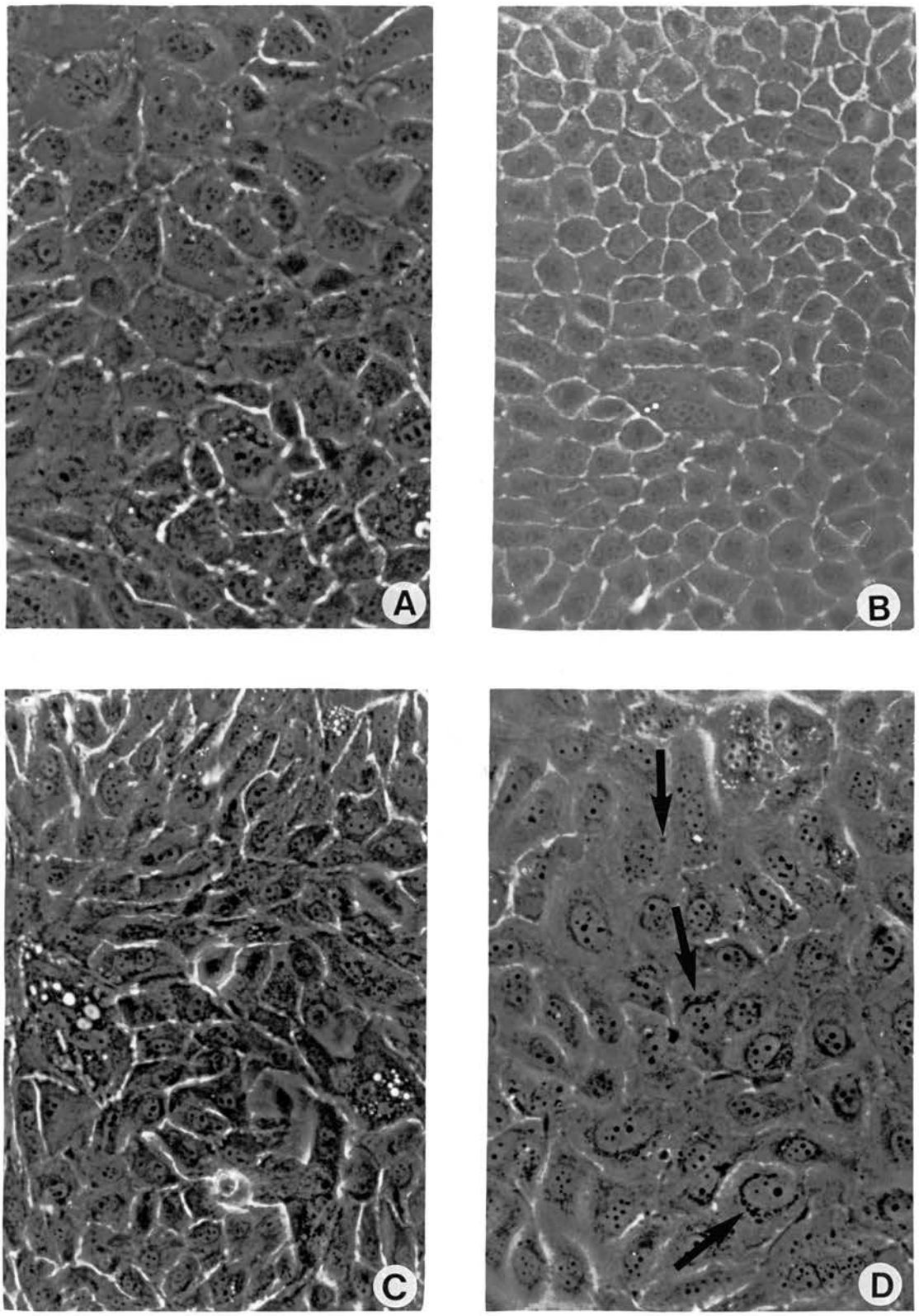
Contact inhibition did not occur in three lines, JS7, JS8 and JS15. Cells started to pile up after 4-5 days of plating and formed a pattern of cord-shaped structures with multilocular features. Shedding of rounded cells or groups of cells into the culture medium was observed.

JS14 cells showed no tendency to pile up, although foci of more crowded cells were noticed when they reached a confluent stage.

One of the most consistent features of all SPA cell lines was the formation of hemicysts (Leighton *et al*, 1969, Synonyms: secretory vesicles, Auersperg, 1969; domes, Pickett *et al*, 1975; blisters, Leigh *et al*, 1984). The cells did not produce hemicysts in sparsely populated cultures, but hemicysts formed after the cells reached confluency. Each hemicyst appeared as a well-circumscribed fluid-filled cavity bounded by a thin

Fig. 4.1A,B,C,D

Shows SPA cultures with epithelial morphology. A, JS14; B, JS15; C, JS8; D, JS7. Note the presence of perinuclear granules in JS7 cells (arrows). Phase contrast, A, x 256; B, x 256; C, x 250; D, x 262.


A
B
C
D

membrane of extenuated cells (Fig. 4.2A & C). Hemicysts were composed of cells which were flat, vacuolated, polygonal and larger in size compared to other cells of the monolayer (Fig. 4.2B & D). Cells of the hemicysts were morphologically similar in all cell lines. Hemicysts demonstrated elevated boundaries and their morphology was alike in the four cell lines. Extensive piling up of hemicyst cells was observed only in cell lines which showed loss of contact inhibition. The piled up cells showed a well organised pattern of acini-like or multilocular structures (Fig.4.3). In cultures of JS14, JS15, JS8, and JS7 hemicysts appeared at passage levels, 5, 15, 20, and 35 respectively. In JS14 hemicysts remained until passage 15 after which they started to decrease in size and number. In cultures of JS7 and JS8 cell lines the hemicysts became larger and more obvious at higher passage level and were still observed at passage 140.

The in vitro growth crisis, which commonly occurs during transformation of cells in culture, was not observed in any of the epithelial cell cultures established from sheep pulmonary adenomatosis.

The growth rate of the JS14 cell line decreased gradually from passage 25 and growth stopped at passage 33. In contrast, the growth rates of JS7 and JS8 began to increase at passage 30. The increase in growth rate was reflected by an ability to raise the splitting ratio (1:10) which increased in subsequent passages to

Fig. 4.2A,B,C,D

Phase contrast photomicrographs of hemicysts in confluent cultures of JS7 (Fig. A & B), and JS14 (Fig. C & D) cells grown on a plastic tissue culture flask. The plane of focus is on the top of the hemicysts in Fig. B & D and on the main monolayer in Fig. A & C. x 128

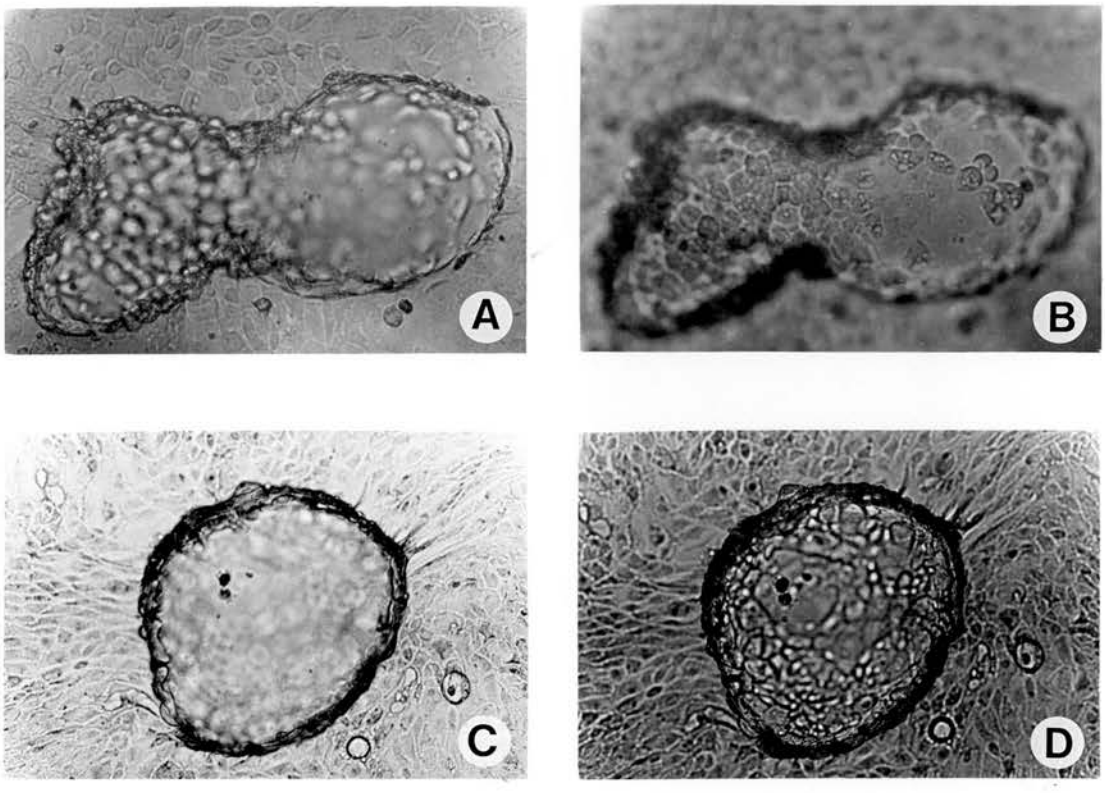
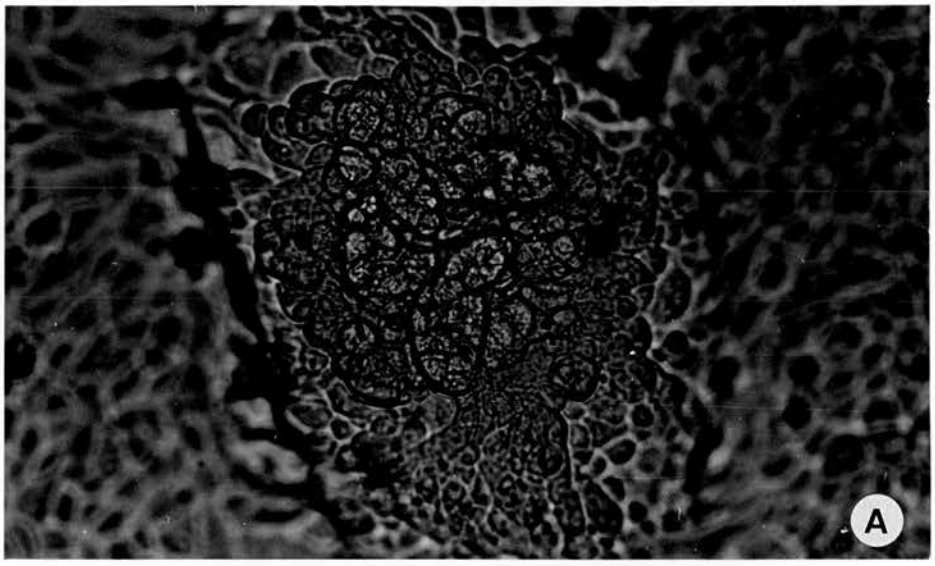
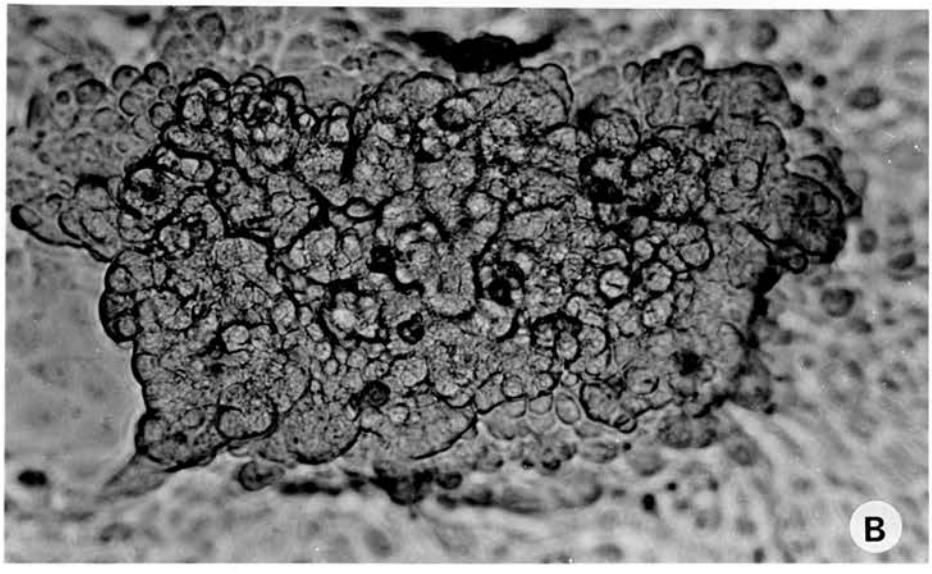

A
B
C
D

Fig. 4.3A,B

Showing hemicysts in culture of JS7 cells (Passage 131). (A), 6 days old culture. Note the appearance of multilocular structures in the hemicyst. (B), the same hemicyst as in A, but on day 7. Note extensive development of multilocular structures. Phase contrast. x 240


A


B

as high as 1:20 after passage 50 and 1:100 after passage 80. However, neither JS7 nor JS8, which were established as permanent cell lines, showed gross alterations in morphology even when cultivated for 140 passages *in vitro*.

Content of type II pneumocytes in cultures

Type II pnemocytes when stained with phosphine 3R displayed golden fluorescence as a result of binding of this dye to lamellar bodies. The staining pattern appeared granular or diffuse (Fig. 4.4). Cells of JS7 and JS15 demonstrated type II pneumocytes between 85% to 90%, whereas, JS8 and JS14 did not exceed 8% to 15%.

Plating efficiency

The plating efficiency of each cell line was examined at the 20th transfer and is shown in Table 4.1. The data indicate that cell lines JS7 and JS8 attained plating efficiencies of more than 54% which is significantly ($P < 0.001$) higher than those of JS14 and JS15 which showed plating efficiencies of 14% and 31% respectively.

The plating efficiencies were not affected by cell input, except JS15 cell line, in which the plating efficiency increased with increasing cell density.

No differences was found between the plating efficiencies of JS7 and JS8 cell lines even when cells were plated at varying densities.

Fig. 4.4

Photomicrograph of a living cell suspension of JS7 cells stained by Phosphine 3R stain for lipids. Cytoplasmic granules exhibiting a bright-yellow fluorescence are seen in most of the cells. x 256

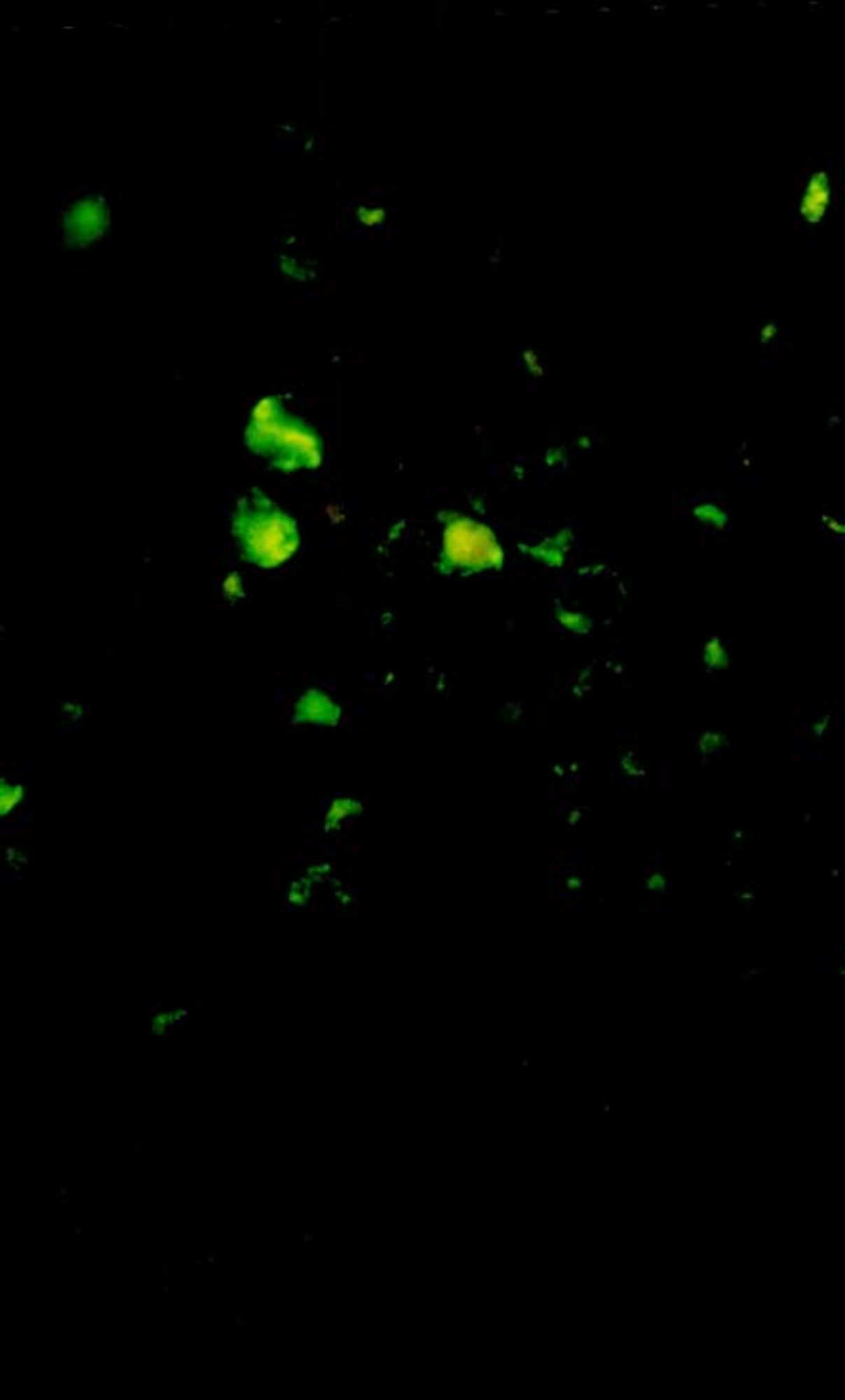


Table 4.1

Plating efficiency on plastic of the four cell lines

Cell line	Plating efficiency (%)		
	100 cells	250 cells	500 cells
JS7	50.3 ± 3.75	54.8 ± 3.66	55.7 ± 0.61
JS8	51.8 ± 2.56	52.4 ± 1.40	54.4 ± 1.57
JS14	12.8 ± 0.85	13.3 ± 0.66	14.2 ± 0.50
JS15	16.5 ± 0.87	27.8 ± 3.76	31.3 ± 2.52

SE± %

Attachment efficiency

The attachment efficiencies of all cell lines are illustrated in Table 4.2. No distinct differences in the attachment efficiencies were exhibited between JS7 and JS8 lines. These two cell lines demonstrated efficiencies much higher than those of the other cell lines, but still JS7, JS8 and JS15 cell lines showed attachment efficiencies markedly higher than that (61%) of JS14 cell line.

Growth kinetics

In the growth curve experiments shown in Fig. 4.5, a lag phase of 24 hours was demonstrated for cells of JS14, JS8, JS15, JS7. These cells increased in number logarithmically until day 4, 6, 7, and 10 respectively. The doubling times were calculated to be 26, 28, 31, and 34 hours respectively.

Saturation density

Large differences were seen in the final cell density reached (Table 4.3). The JS14 line reached a density of 3.4×10^4 cells/cm^2 and formed a confluent monolayer containing relatively large cells. In contrast, JS15, JS8, and JS7 lines continued to grow well beyond this concentration and, respectively, reached final densities representing 8, 11, and 17-fold higher that that of the JS14 cells. At high densities these cell lines formed smaller, more cuboidal cells packed into a single layer, with a tendency to pile up.

Table 4.2

Attachment efficiency of the four cell lines.

Cell line	Attachment efficiency (%) per plate
JS7	88, 83
JS8	91, 88
JS14	64, 59
JS15	74, 72

Fig. 4.5

Growth kinetics of SPA cell lines in culture. Points, represent the mean number of cells in duplicate plates.

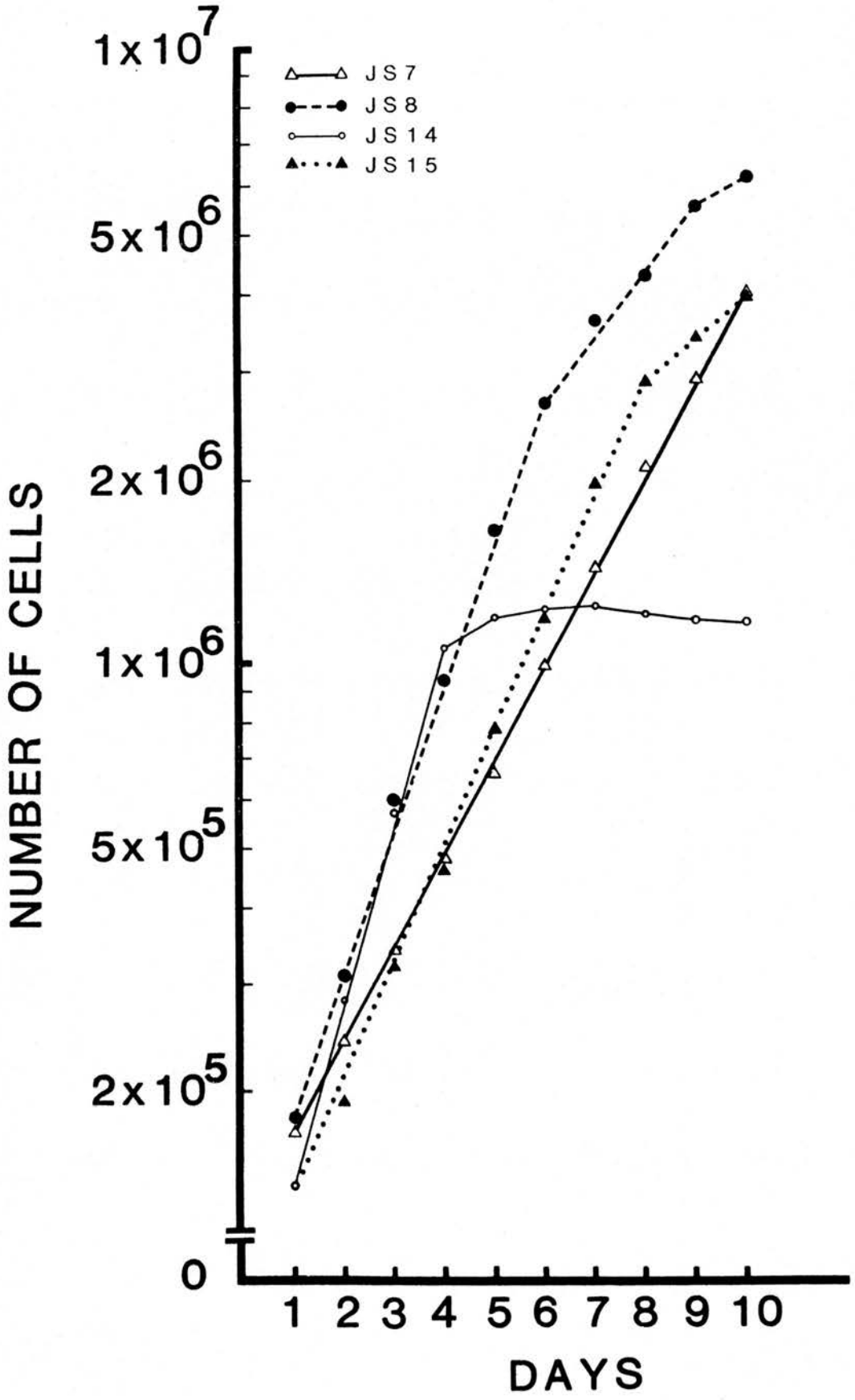

1x10^7
5x10^6
2x10^6
1x10^6
5x10^5
2x10^5
0
NUMBER OF CELLS
JS7
JS8
JS14
JS15
1 2 3 4 5 6 7 8 9 10
DAYS

Table 4.3

Saturation densities of the four cell lines.

Cell line	Saturation density x 10^5/cm²/ flask
JS7	5.9, 5.7
JS8	3.9, 3.5
JS14	0.4, 0.3
JS15	2.8, 2.4

Electron microscopy

The ultrastructural features of the three cell lines (JS7, JS8, JS14) are similar and consistent with those of epithelial cells. The features of each individual cell have remained constant irrespective of their passage history.

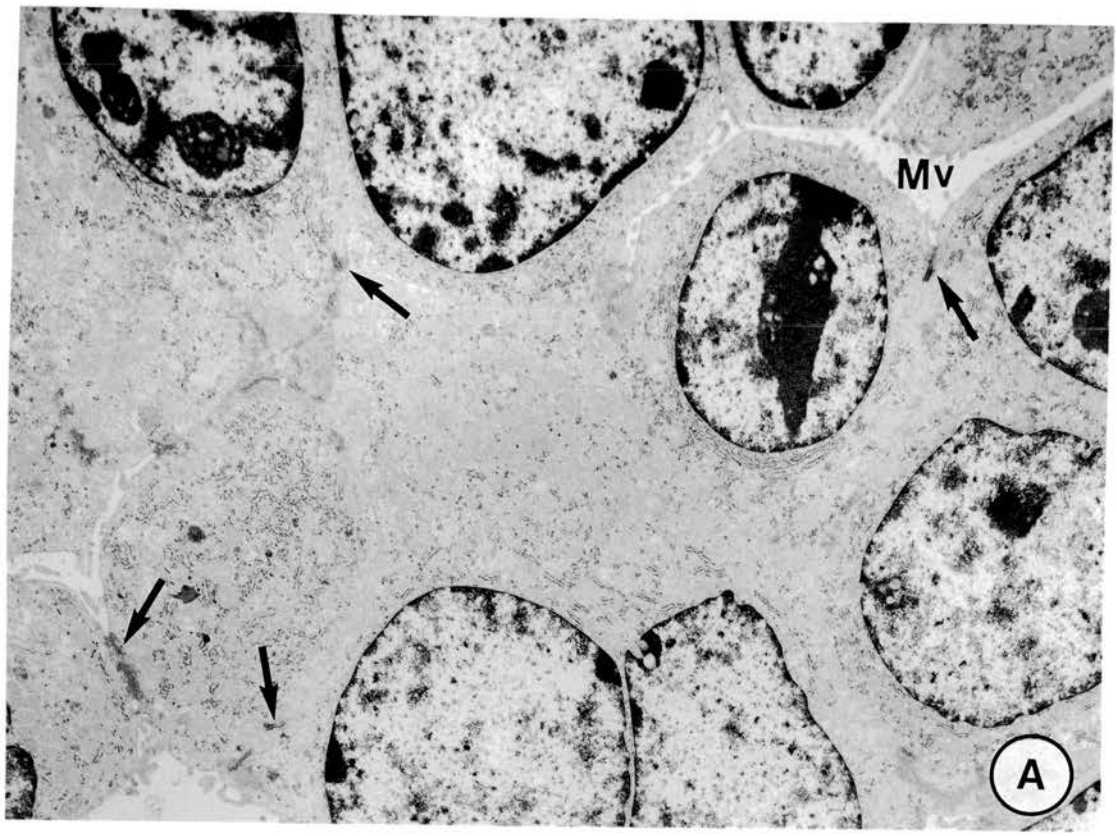
Surface microvilli, distinct bundles of tonofilaments in parallel orientation and desmosomes between cells were observed (Fig.4.6). Golgi apparatus and endoplasmic reticulum were well developed. Mitochondria were numerous and pleomorphic. The nuclei were, in general, round, oval, or irregular with many indentations. One or more prominent nucleoli were seen in each nucleus. Ribosomes were abundant, either free in the cytoplasm or bound to the membranes of endoplasmic reticulum.

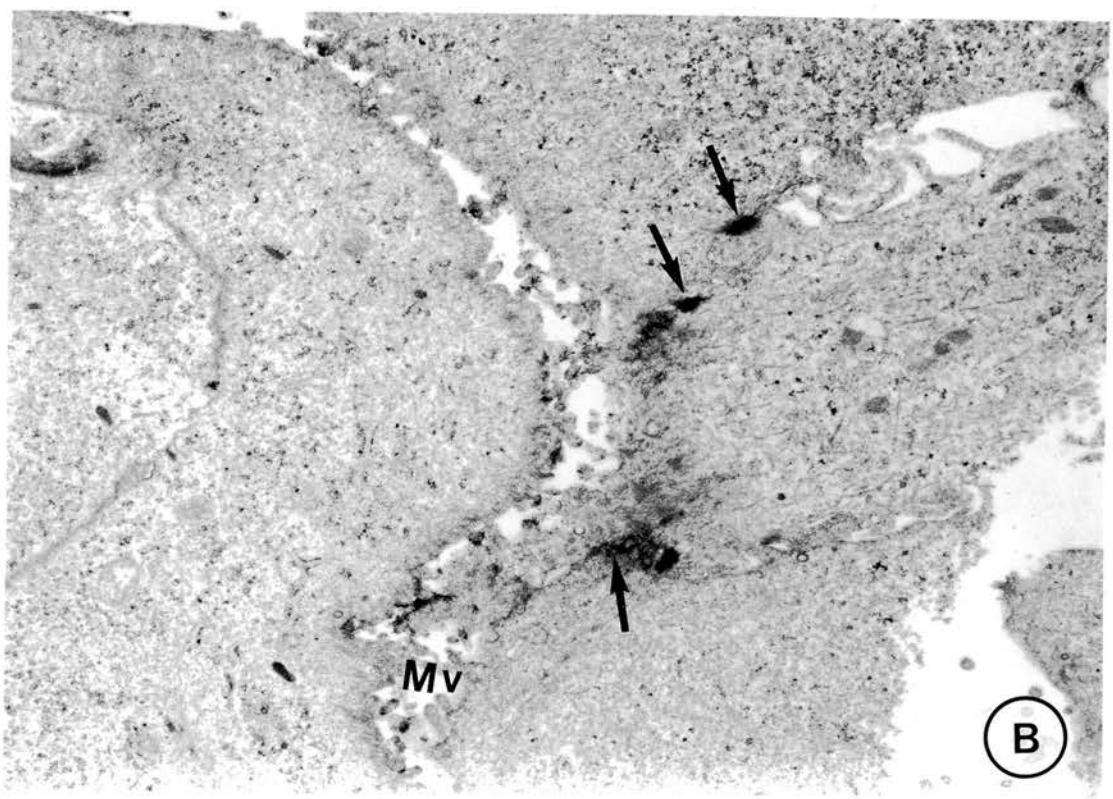
Membrane-bound cytoplasmic vesicles, housing smaller vesicles or bodies of variable size and shape, were often seen in many cells.

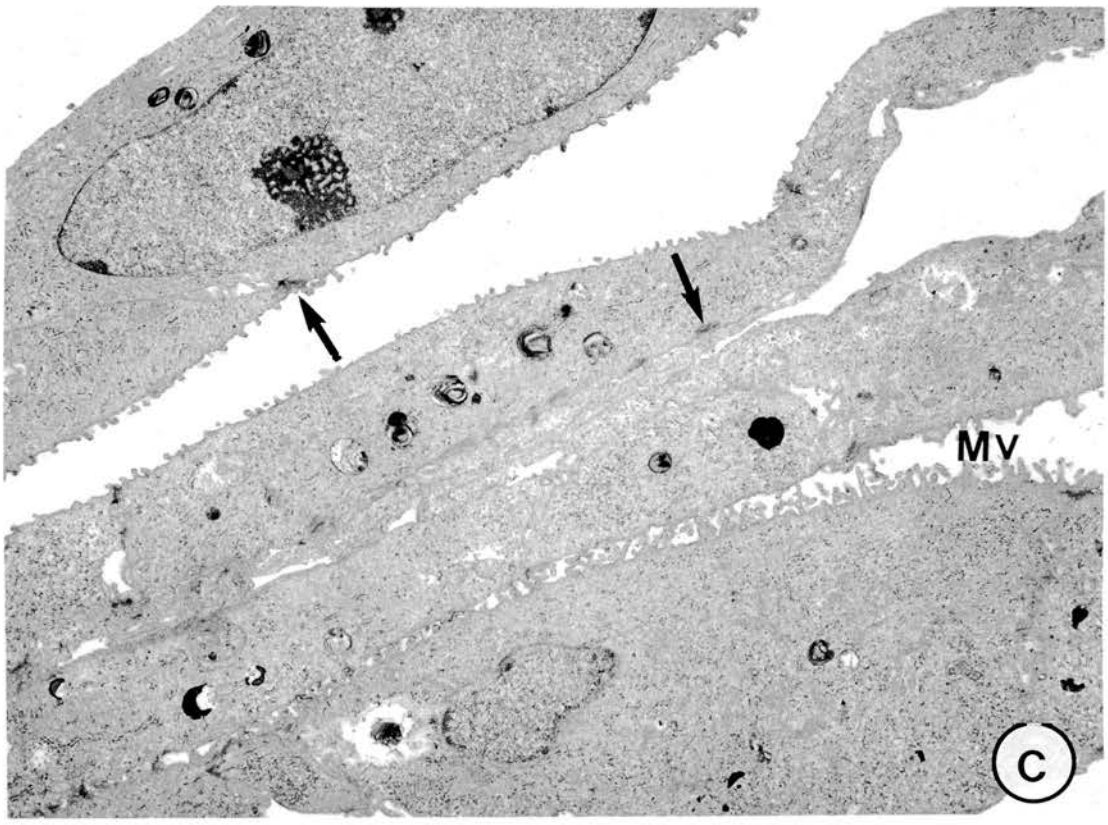
Lamellar bodies characteristic of type II pneumocytes were observed in various numbers in most cells of line JS7 (Fig.4.6C). They were mostly rounded and variable in size. The lamellar bodies present in these cells had two different appearances (Fig. 4.7). Some presented a lamellar structure with an electron-lucent central area, while others had a dense homogeneous morphology. Lamellar bodies displaying an intermediate morphology were also present. The lamellar bodies seen in

Fig. 4.6A,B,C,D

Electron micrographs of (A),JS8; (B),JS14; and (C&D),JS7, showing markers of epithelial cell. Note microvilli (Mv) on the apical surface of cells; desmosomes (arrows) join the cells to one another; and bundles of tonofilaments (Tf) appearing in (D).
(A), x 6000; (B), x 15000; (C), x 6000; (D), x 20000.


Mv
A


Mv
B


Mv
C

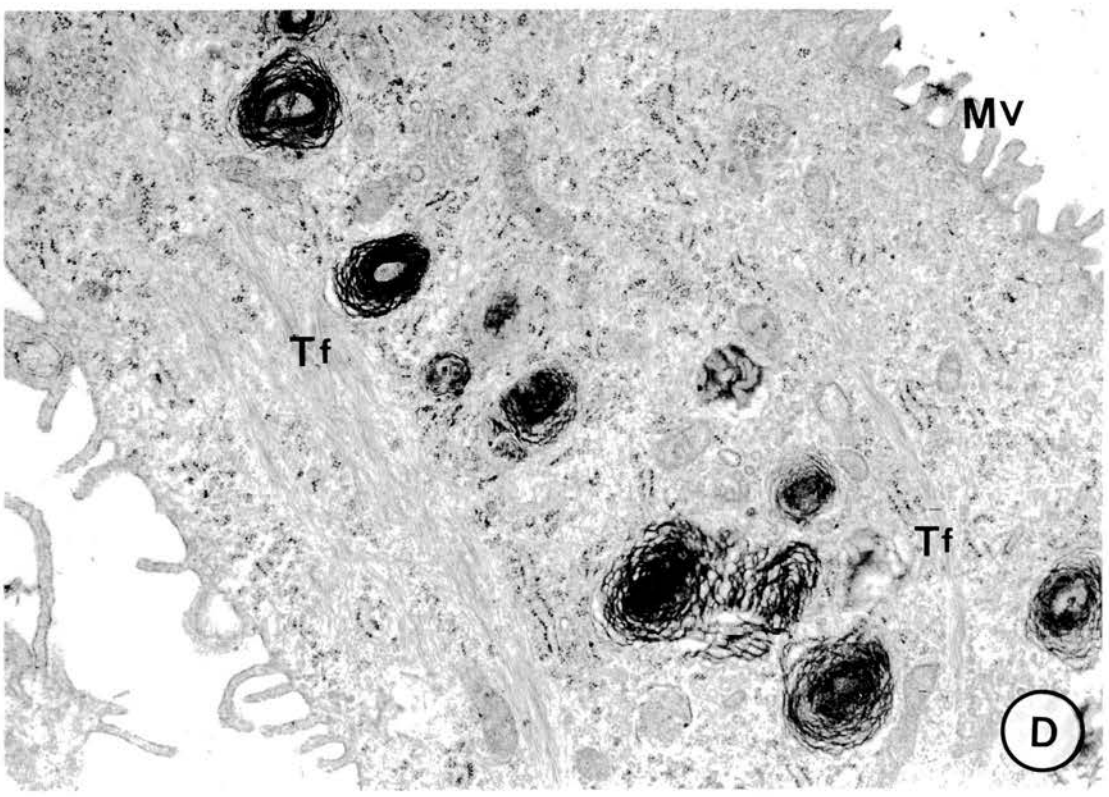
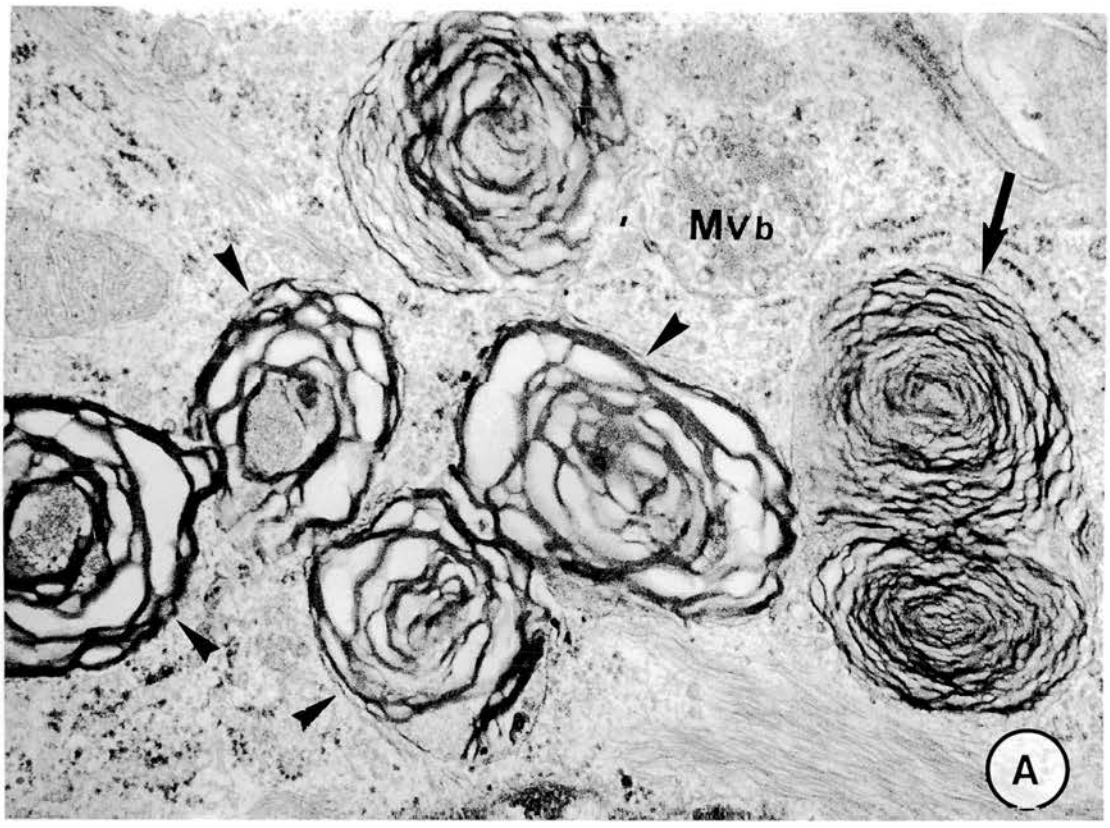
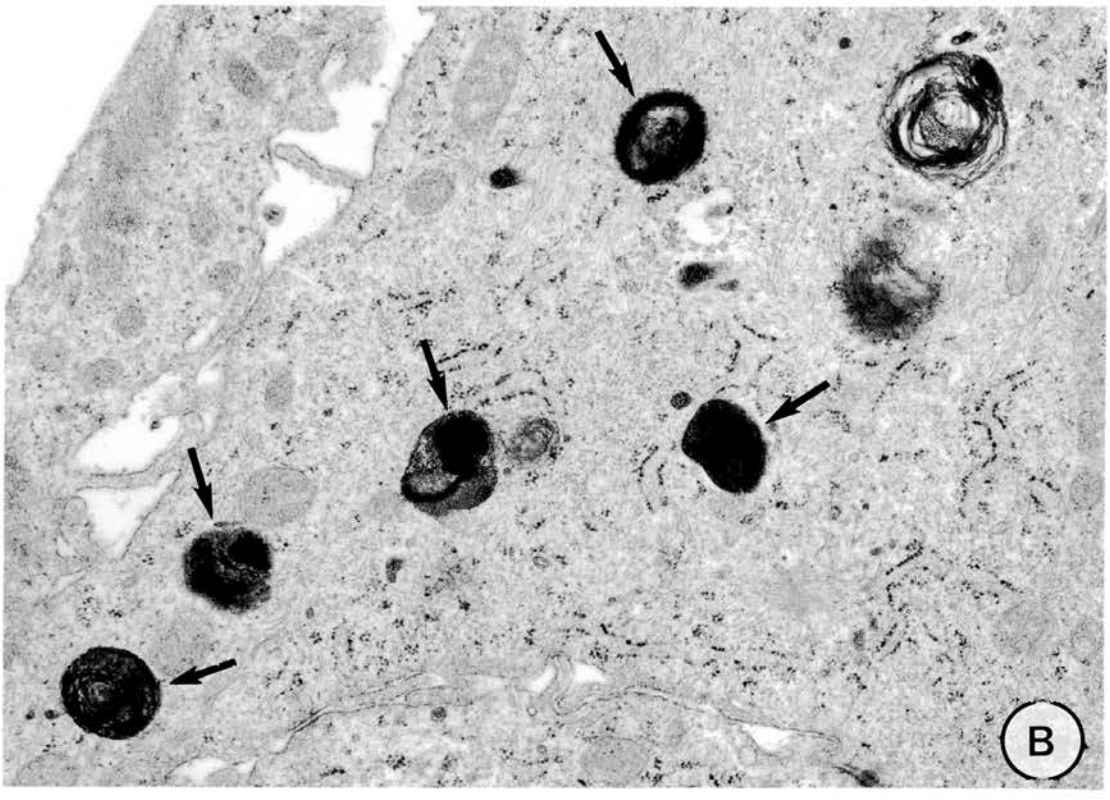

Mv
Tf
Tf
D

Fig. 4.7A,B

Electron micrographs of type II cells in the JS7 cell line. Numerous lamellar bodies are present in the cytoplasm of each type II cell. The morphology of these varies, some have a homogeneous appearance (B, arrows), and others, have a lamellar structure (A, arrow heads). Intermediate forms are present (A, arrows), as are multivesicular bodies (Mvb), which are regarded as a precursor of the lamellar body.

A, x 40000; B, x 16000.


MVb
A


B

cells when examined at passage 6, 25 and 37 were devoid of a limiting membrane, but at passage 112, lamellar bodies with or without limiting membranes were seen (Fig. 4.8). Multivesicular bodies (Fig. 4.7A) were commonly seen in JS7 cells but neither JS8 nor JS14 cells contained these structures.

Glycogen granules arranged in aggregates were a common feature of JS7 cells (Fig. 4.9), but were seen in only a few cells of JS8 and not at all in JS14.

No virus particles was found in any of the cell lines despite extensive searching.

Chromosome analysis

The cell lines were examined for the distribution of chromosome numbers at the 20th passage. Sixty-six good metaphases of each cell line were analysed. As illustrated in Fig. 4.10, all cell lines were found at this stage to be aneuploid. In all of the metaphases that were examined there was no loss of any of the six large metacentric chromosomes characteristic of the ovine karyotype. However, in hyperdiploid metaphases, the 3 pairs of metacentrics increased 2 to 4 fold. In hypodiploid cells the loss was restricted to the acrocentric and telocentric chromosomes but the metacentric chromosomes remained unchanged.

Line JS7

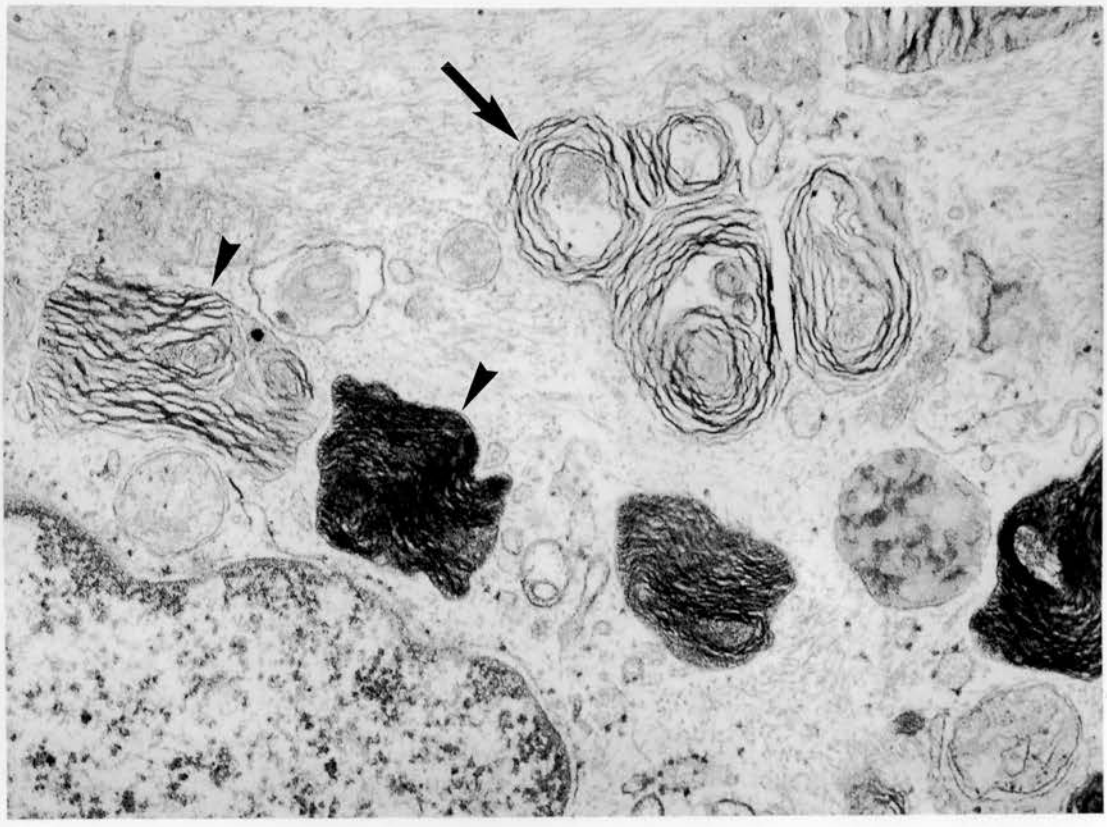
The number of chromosomes in these cells varied from 47 to 178 with a bimodal chromosome number of 85 and 90 in 15% and 14% respectively of the metaphases

Fig. 4.8

Electron micrograph of a portion of a type II cell in the JS7 cell line after 112 passages *in vitro*, illustrating lamellar bodies with limiting membrane (arrow heads) and without limiting membrane (arrows). x 20000.

Fig. 4.9

A portion of a cell in the JS7 cell line showing the presence of a large number of glycogen granule clusters (arrows).
x 16000.



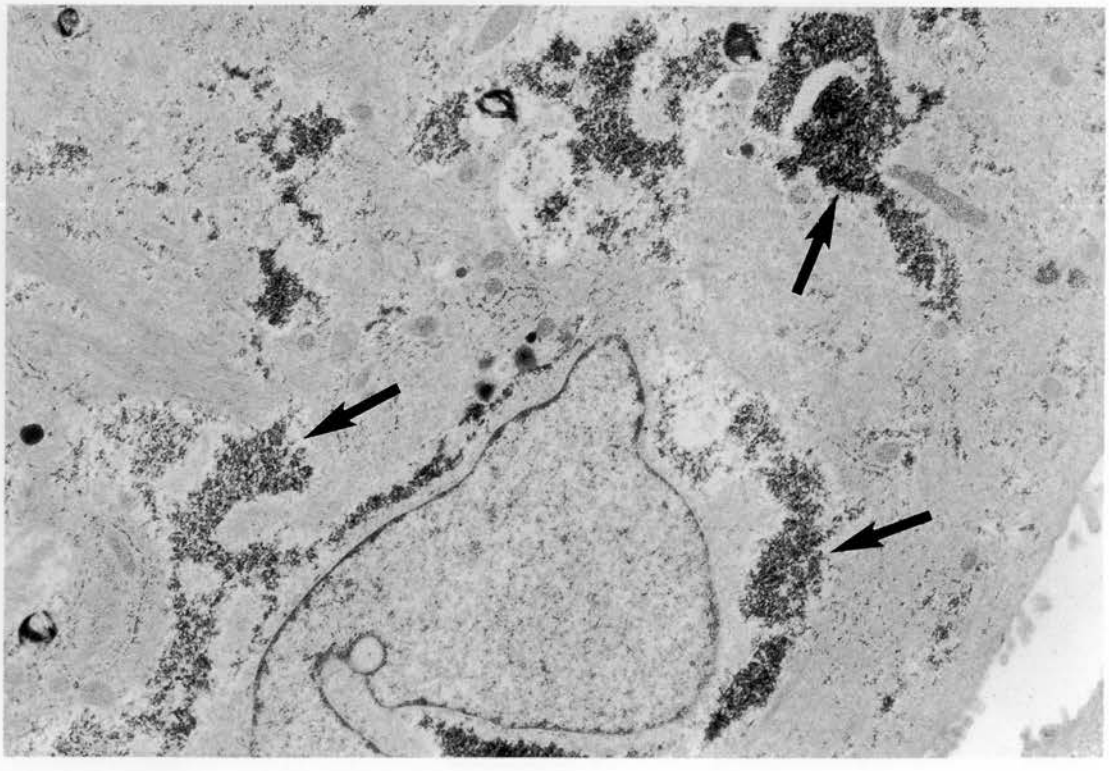
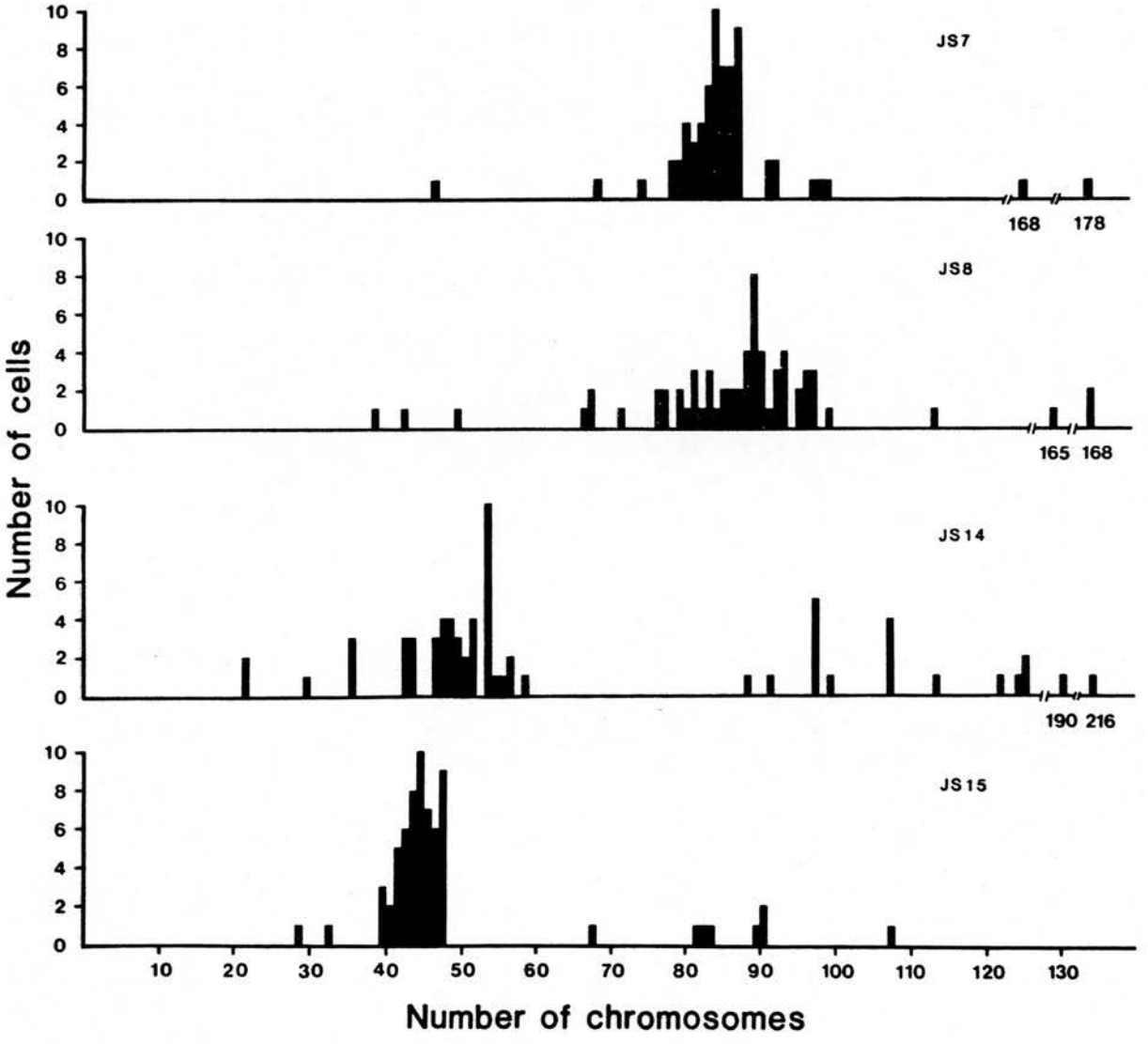


Fig. 4.10

Distribution of karyotypes in passage 20 of the indicated SPA cell lines.


JS7
JS8
JS14
JS15
168
178
165
168
190
216
Number of cells
Number of chromosomes
0
2
4
6
8
10
10
20
30
40
50
60
70
80
90
100
110
120
130

examined. A large marker chromosome consisting of a long and short arm (Fig. 4.11) was identified as an unusual chromosome in 52% of the total metaphases analysed. A dicentric chromosome was also identified in some metaphases (Fig. 4.12) with a frequency of 5% of the metaphases counted. This chromosome is composed of three segments with the part in the middle appearing larger than the other two segments which also look different in size. The presence of these two abnormal chromosomes is not related to a particular metaphase i.e. they could be found in any metaphase regardless of the number of chromosomes.

Line JS8

The chromosome number ranged from 37 to 168 with a modal number of 90 chromosomes consisting of 12% of the 66 metaphases examined. No large marker chromosome was found in metaphases of this cell line. However, unusual double-minute chromosomes (DMs) were observed. When the 66 metaphases were surveyed for the presence of DMs, they were observed in 25 metaphases (36.4%) examined. These chromosomes were seen only in those metaphases containing 80 to 168 chromosomes, and 5 of the 8 metaphases that constituted the modal number of this cell lines contained DMs. The number of DMs varied considerably among the cells, ranging from 2 to more than 100 per cell. They appeared as round, small or large single or double dots (Fig. 4.13). The smaller sizes stained much lighter than the larger ones.

Fig. 4.11A,B

Metaphases in JS7 line. (A), exhibits a large chromosome with a long and short arm (arrow); (B), submetacentric chromosomes (arrows).


A


B

Fig. 4.12

A metaphase in JS7 cells showing a dicentric chromosome. Arrows indicate the two constrictions.

Fig. 4.13

A metaphase in JS8 cell line. Note the minute chromosomes occurring as single or double dots.

Line JS15

This cell line was hypodiploid, and had a modal chromosome number of 45 and a range of 29 to 108 chromosomes. The modal number was seen in 21% of the 66 metaphase spreads examined. The karyotype analysis showed unusual dicentric chromosomes in seven of 66 (11%) metaphases examined (Fig. 4.14A). In hypodiploid metaphases one or two extra metacentric chromosomes (Fig. 4.14B) were identified in 37 metaphases of the 57 (65%) examined.

Line JS14

The number of chromosomes in this cell line varied from 22 to 133 per metaphase and demonstrated a diploid modal number of 54 chromosomes at a frequency of 15% of the total metaphases examined. No marker chromosome has been seen in this cell line.

Fig. 4.14A,B

Metaphases in JS15 cell line. (A), showing a dicentric chromosome (arrow), and (B), showing a metaphase with eight metacentric chromosomes (arrows).

A

B

B). GROWTH OF CELLS IN SOFT AGAR

INTRODUCTION

In the course of this Chapter, it was demonstrated that the SPA cell lines showed several features different from normal type II pneumocytes that were consistent with a transformed cell. Following the pioneering work of MacPherson and Montagnier (1964) and also Stoker (1968), Stiles *et al* (1976) showed that growth in agar is one of the most important *in vitro* indicators of transformation. It was thus important to assess the ability of SPA cells to grow in soft agar, and to determine whether this feature correlated with tumourigenicity in the nude mouse.

Although the original soft agar technique supports the growth of many cells, some transformed cells, especially primary tumour cells, need additional promoters to achieve better conditions for their growth, and to enhance the number and size of colonies.

Some investigators obtained better growth of tumour cells by substituting agarose, which lacks polyanions, for agar (Jones *et al*, 1976). Likewise, supplementing media with pig serum or insulin improved the growth of cells in agar (Clarke *et al*, 1970; Otsuka, 1972). Other investigators found that the macrophages that are present in tumour cell suspensions may either stimulate or inhibit the growth of cells, depending on the type of tumour cells (Hamburger *et al*, 1980; Hamburger

and Salmon, 1980). However, Courtenay and Mills (1978) reported that the addition of a combination of feeder cells and August rat erythrocytes stimulated the growth of tumour cells when incubated in an atmosphere of low oxygen. This technique is now widely accepted because it has increased the cloning efficiency of many tumour cells.

Epidermal growth factor (EGF) is a polypeptide hormone, originally isolated from the male mouse submaxillary gland (Cohen, 1962). It is a potent mitogen for a number of cell types in culture (Turkington, 1969; Stoker et al, 1976; Gospodarowicz et al, 1978; Osborne et al, 1980) and has been shown to stimulate the growth of quiescent cells in culture, which is reflected by increased DNA synthesis. In addition, EGF has been reported to increase clonogenicity as well as the size of colonies produced by tumour cells in agar (Pathak et al, 1982)

In view of these observations, all of the above reported methods were attempted in the present study.

MATERIALS AND METHODS

Cells

SPA cell lines (JS7, JS8, JS14, and JS15), dog mammary carcinoma (Norval et al, 1984) and normal bat lung (NBL12) cells (Chapter 6) were used in this study. In addition, suspensions of SPA tumour cells from which macrophages had been depleted, as described in Chapter 3 were also examined.

Epidermal growth factor

Epidermal growth factor (EGF), derived from male mouse submaxillary glands, was used in the present study and was purchased from Sigma Chemical Company. Stock EGF was prepared in phosphate buffer saline at a concentration of 10 ng/µl.

Conditioned medium

Conditioned medium collected from the same SPA lines, as well as NBL12 and dog mammary carcinoma cultures, was clarified by centrifugation at 10,000xg for 30 minutes, filtered and used immediately. These conditioned media were used both at full strength and as 10% in the agar medium supplemented with the indicated fetal bovine serum.

Courtenay and Mills Technique

The technique used in the initial investigation was based on that of Courtenay and Mills (1978). The essential features of the procedure are:-

the application of low oxygen concentration (5%) and the addition of feeder cells such as August rat erythrocytes or irradiated autologous tumour epithelial cells (Fig. 4.15A).

August rat erythrocytes were collected in heparin from August rats bred at the Moredun Research Institute Small Animal Unit. Freshly prepared erythrocytes were washed three times in cold phosphate buffered saline, incubated in a water bath at 44°C for one hour, diluted 1/2 in medium, then mixed with the agar medium at a final concentration of 10% (v/v). SPA tumour cell suspensions were heavily irradiated with 10,000 rads of gamma irradiation from a cobalt-60 source shortly before adding them to the agar layer. They were used at a final concentration of 10^4 cells/dish.

Various modifications to the above procedure are detailed below.

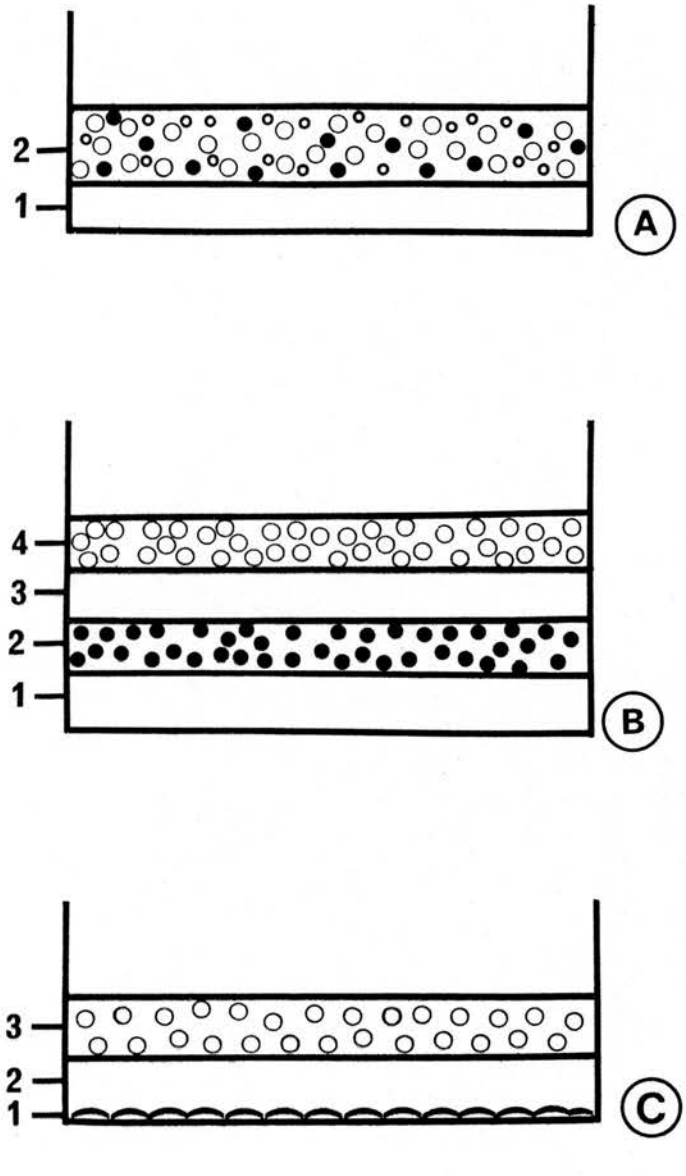
First, 10^5 NBL12 or dog mammary carcinoma cells suspended in 2 ml of 0.3% agar medium were layered on to a 0.6% preset agar base layer, which was then covered with 1ml of 0.3% agar (Fig. 4.15B). Secondly, monolayers prepared from the autologous cell line or NBL12 or mammary carcinoma, were covered 24 hours later with 2ml of 0.6% agar medium (Fig. 4.15C). The two feeder layers were overlaid with 2ml of 0.3% agar medium containing 2×10^5 tumour cells.

Fig. 4.15A,B,C

Schematic representation of different layers. A1, 0.6% agar layer; A2, 0.3% agar containing irradiated autologous tumour cells, August rat erythrocytes and the tumour cells under test.

B1, 0.6% agar; B2, 0.3% agar containing feeder cells (as described in materials and methods); B3, 0.6% agar; B4, 0.3% agar containing tumour cells under test.

C1, cell monolayer; C2, 0.6% agar; C3, 0.3% agar containing tumour cells under test.


2
1
A
4
3
2
1
B
3
2
1
C

Gas phase

Two gas mixtures were used; 5% O_2, 5% CO_2,90% N_2 (obtained from British Oxygen Company), and 5% CO_2 in air. Cultures grown in the former gas mixture were kept enclosed in a modular incubator chamber (Fig. 4.16) purchased from Flow Laboratories.

Recloning of colony cells

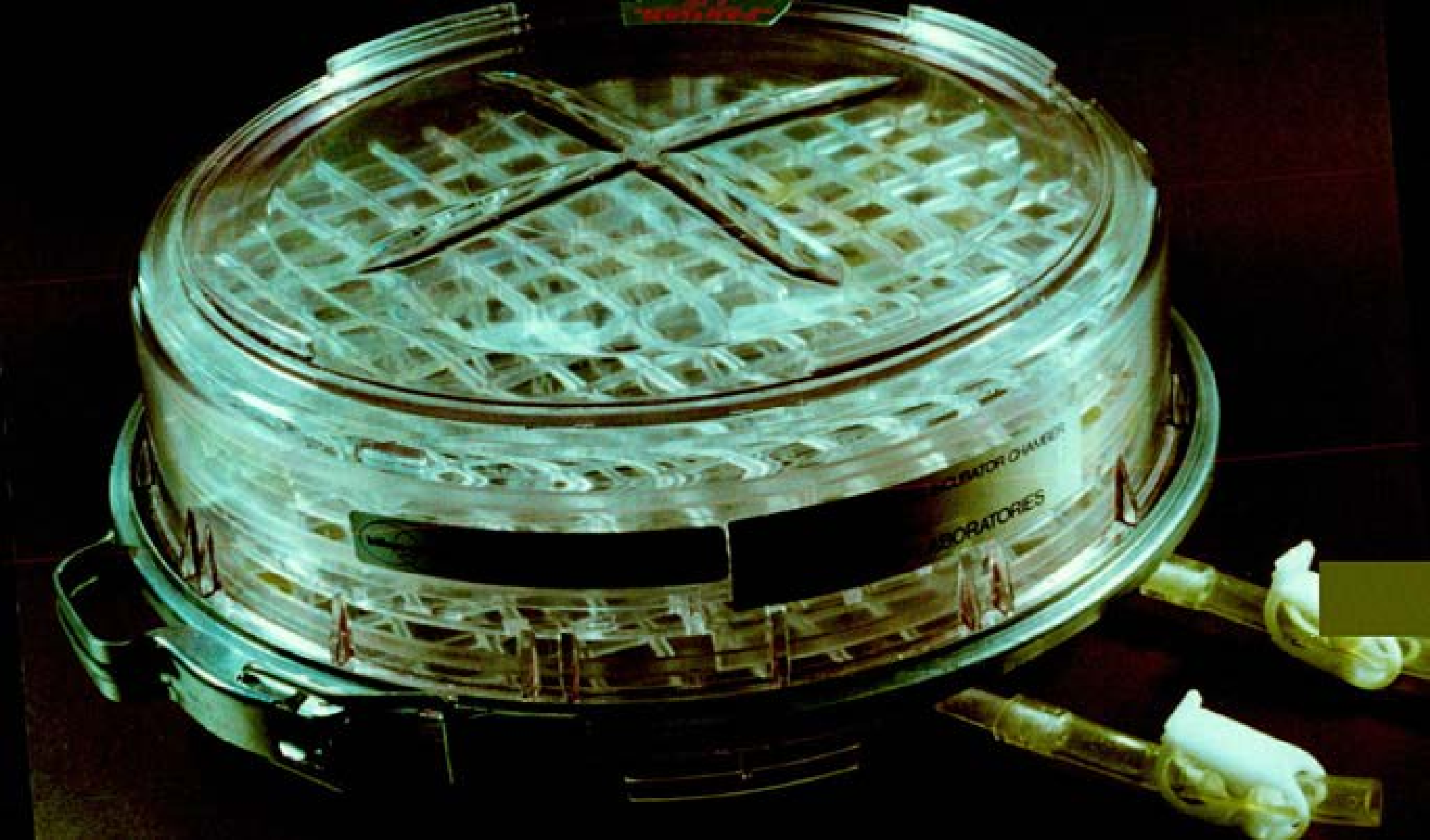
Individual colonies were recloned by aspirating the colony with a pipette containing a drop of medium with the aid of a binocular microscope (x 25 magnification). The individual colonies were directly plated in 24-well culture plates (Falcon), then covered with 2ml of 0.3% agar medium. Cultures were fed every four days with 1ml of the overlay medium. Cultures initiated from each individual colony were reassayed in agar to compare their cloning efficiency with that of the parent cells.

Histology

To examine the morphology of the colonies and their constituent cells, colonies were fixed, after three week's incubation, by gently adding 4ml of Bouin's fixative and allowing the plates to stand at 4°C for 24 hours. The fixative was then removed by aspiration with a Pasteur pipette. Blocks containing colonies were carefully cut out with a fine scalpel and placed in tissue cassettes. The blocks were transferred to 96% (v/v) alcohol and processed routinely for haematoxylin and eosin staining.

Fig. 4.16

A modular incubator chamber in which cultures were enclosed.



Transmission electron microsopy

After a 14-day incubation period, colonies in the upper layer were fixed for 24 hours at 4°C with 3ml of a freshly prepared solution of 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.2. Colonies were scored under a dissecting microscope, and small blocks containing several colonies were carefully cut out, then prepared for transmission electron microscopy as described in General Materials and Methods.

RESULTS

Evaluation of early experiments

Using the Courtenay and Mills (1978) technique, none of the SPA cell cultures showed evidence of producing colonies when taken directly from cell suspensions or after growth *in vitro* for less than 16 passages, even when plated at a high cell density (10^6 cells/plate).

Furthermore, various modifications of the Courtenay and Mills (1978) procedures were investigated but were equally unsuccessful. For example, neither keeping autologous macrophages in the cell suspension nor their removal showed any effect and did not stimulate the cells to grow in agar. Similarly, none of the various systems of feeder layers or conditioned media supported the growth of SPA cells in agar. Cells which did not grow in agar also failed to grow in agarose. Likewise, supplementing the medium with pig serum or insulin did not promote the growth of cells in agar or agarose.

SPA cells were first observed to grow in agar at passages greater than 16. Consequently, the ability of the four cell lines (JS7, JS8, JS14, JS15) to grow in soft agar was examined further. Also the possibility that EGF might stimulate their growth was examined.

Experiment 1

Experimental design

Cells of the above named cell lines (passage 20) were plated at a density of 10^5 cells per plate (as described in "General Materials and Methods") in medium lacking or containing 50 ng/ml EGF in both the upper and lower agar medium. Colony numbers were determined after incubation for three weeks and expressed as the mean number of colonies from triplicate cultures (±SE) or as a colony forming efficiency (% = number of colonies x 100/ number of cells plated).

Results

Growth and colony development

Discrete colonies became evident within 4-5 days of plating and, thereafter, the number of colonies increased to a peak by 7 to 10 days. By this time, cells which failed to produce colonies were completely autolysed. Most of the colonies slowly increased in size and attained a maximum size 3 weeks after incubation. A week later, with all cell lines, the majority of colonies tended to show necrosis and had usually died by the end of the 4th week after plating. However, colonies in medium supplemented with EGF appeared earlier and started to degenerate faster.

Colony morphology

Colonies in medium containing or lacking EGF were small, spherical and compact with or without uniform boundaries (Fig. 4.17).

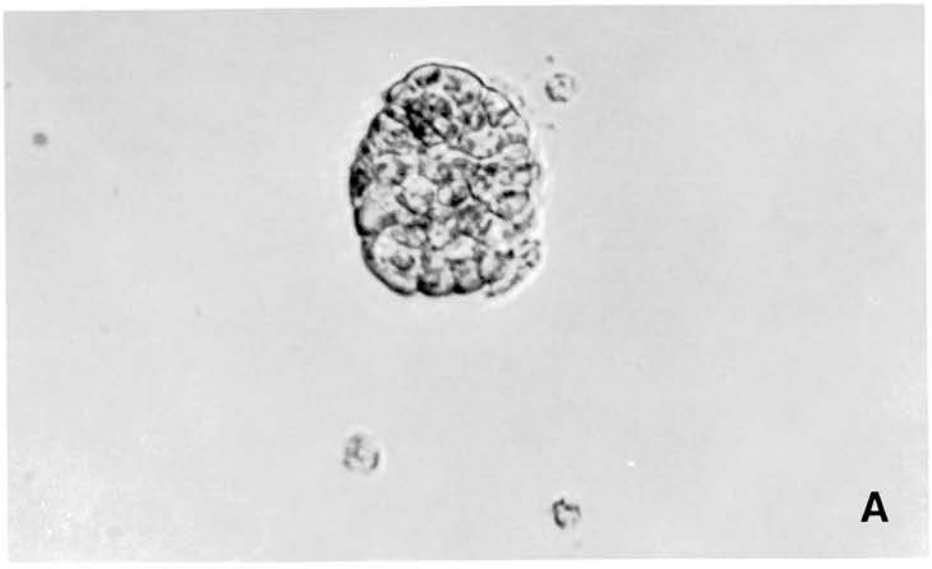
At higher passage levels, two morphological types of colonies were generally distinguished, both in medium lacking or containing EGF. These were either vacuolated or non-vacuolated (Fig. 4.18), but both types were composed of tightly packed cells. In vacuolated colonies, the vacuoles were noticed as early as 24 hours after plating. The non-vacuolated colonies demonstrated smooth boundaries. By the 14th day the two variant morphologies were maintained, but the vacuolation increased in vacuolated colonies, with some colonies forming blisters (Fig. 4.19A), or in a few cases the whole colony appeared as if its centre was occupied by a space (Fig. 4.19B). The non-vacuolated colonies formed structures similar to those produced by monolayer cultures when they started piling up (Fig. 4.19C&D).

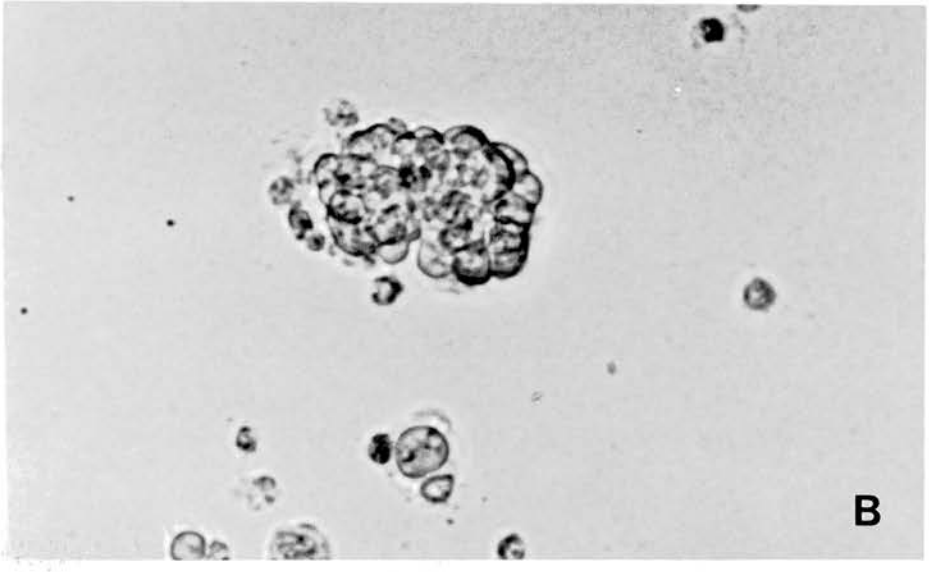
Cloning efficiency

Results shown in Table 4.4 indicate that the range of agar cloning efficiency in the absence of EGF varied from 0.01% to 0.51%. In all cases, it can be seen that a concentration of 50 ng/ml EGF increased the mean colony numbers of these cells by 2 fold to more than 8 fold and enhanced their growth.

Fig. 4.17A,B,C

Showing 21-day-old colonies produced by SPA cell lines (passage 20) in agar. A, JS8; B, JS7; C, JS15, x 288.


A


B

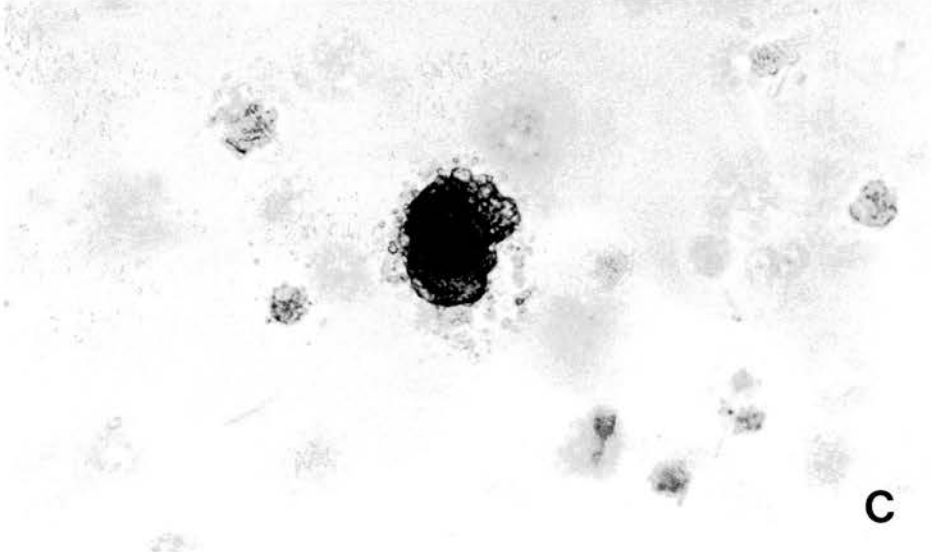
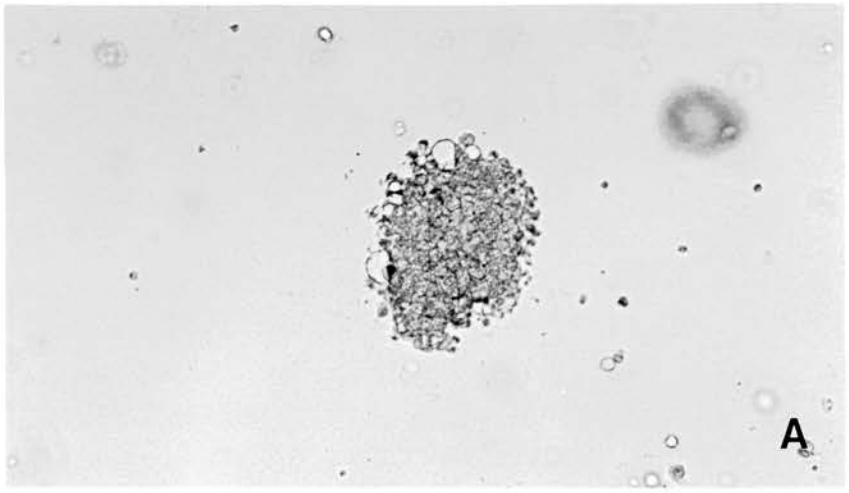
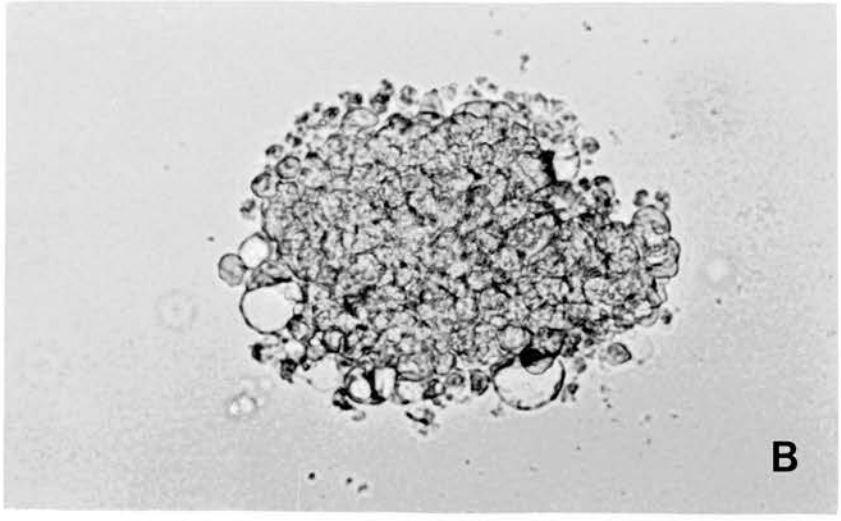
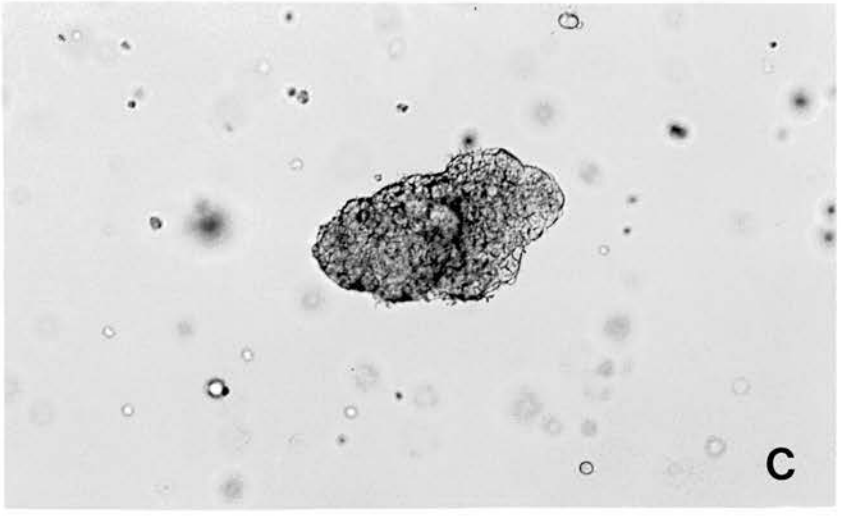

C

Fig. 4.18A,B,C,D

A view of 12-day-old colonies grown in agar from JS7 cells (passage 57). A, a colony showing vacuoles; B, High-power of the colony depicted in A. C, a non-vacuolated colony; D, High-power of the colony shown in C. A, x 102; B, x 218; C, x 102; D, 205.


A


B


C

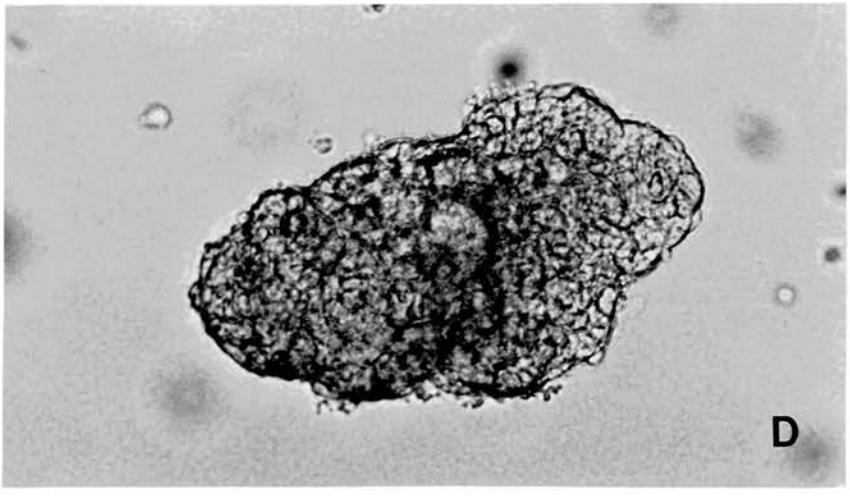
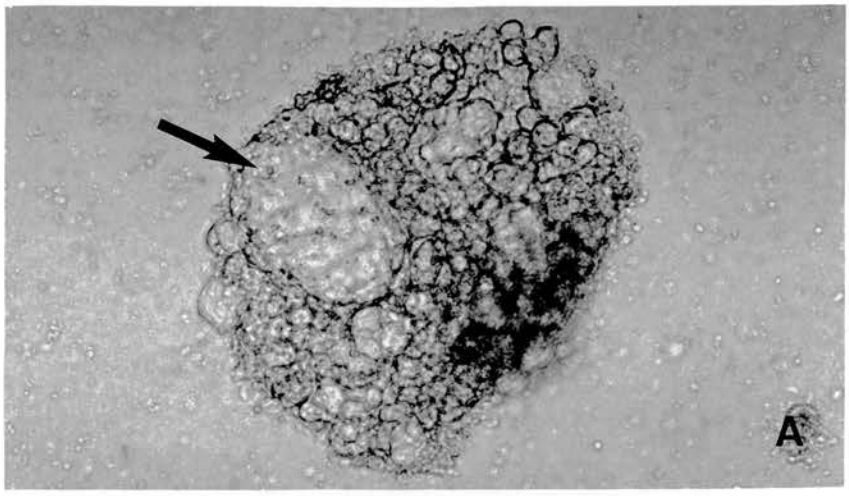
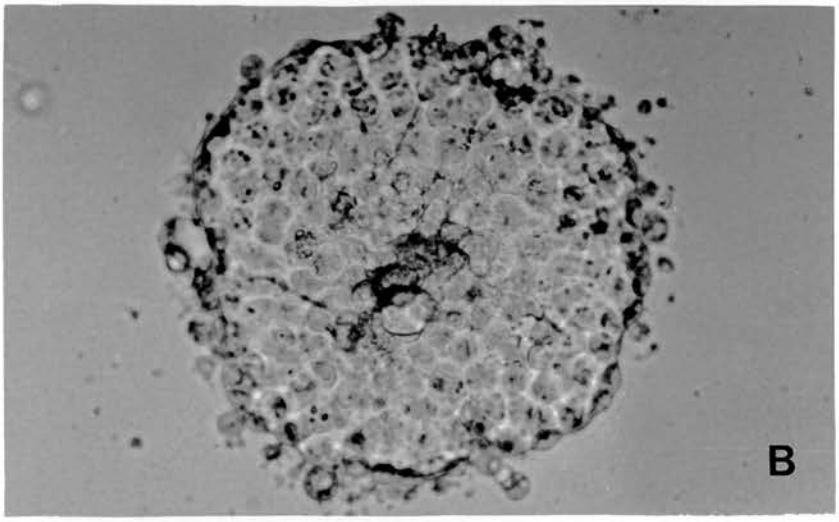
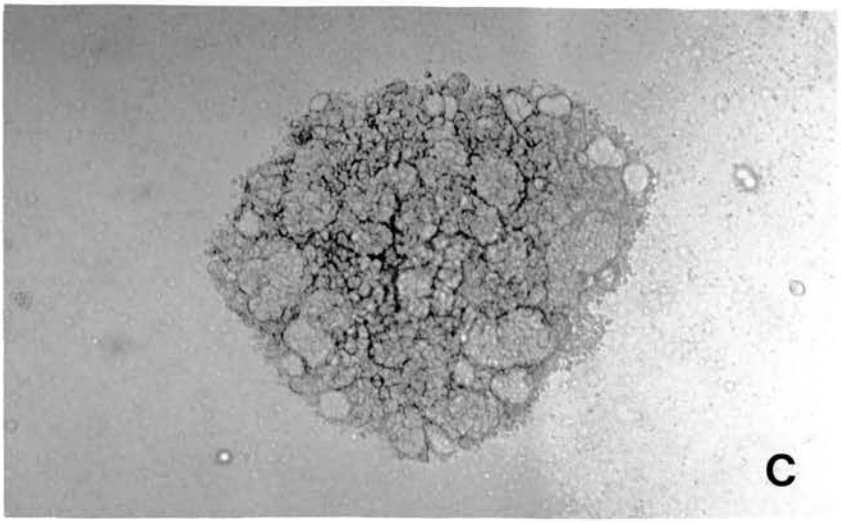

D

Fig. 4.19A,B,C,D

Morphological appearance of JS7 cell colonies after 14 days in agar. A, a colony containing a blister (arrow); B, another colony with apparent space in centre; C and D, depicting structures similar to those induced by cells in liquid culture when piling up. A, x 211; B, x 224; C, x 112; D, x 211.


A


B


C

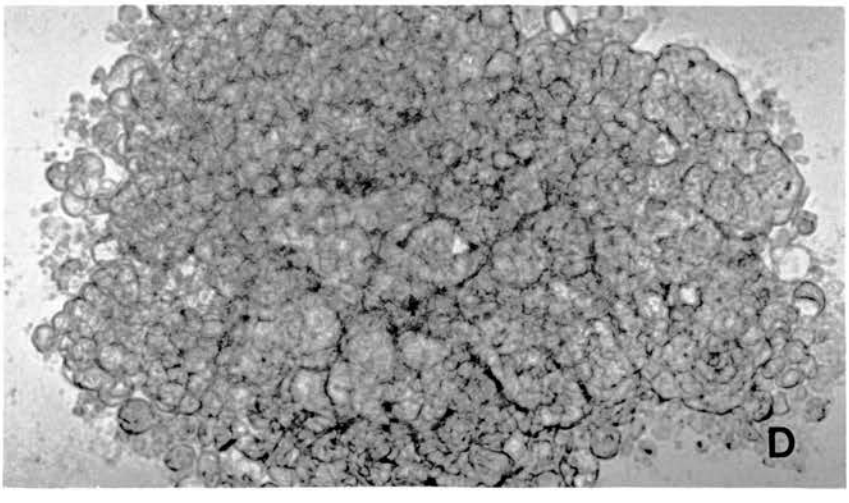

D

Table 4.4

Colony-forming efficiency of four different SPA cell lines

Cell line	Growth in agar	Colony number -EGF	Colony number +EGF
JS7	+	513±26.6 (0.51)	4084±232 (4.10)
JS8	+	4.7±0.88 (0.01)	15.3±0.88 (0.02)
JS14	-	0	0
JS15	+	17.7±1.2 (0.02)	45.7±5.36 (0.05)

Figures in parentheses indicate cloning efficiency.

Experiment 2

Effect of EGF concentration on colony-forming efficiency of JS7 cells

Introduction

Studies with culture monolayers have indicated that the stimulatory effect of EGF is dose-dependent (Osborne et al, 1980). Therefore, an experiment was designed to find out if the stimulation of colony formation in soft agar by EGF also is dose-dependent.

Experimental design

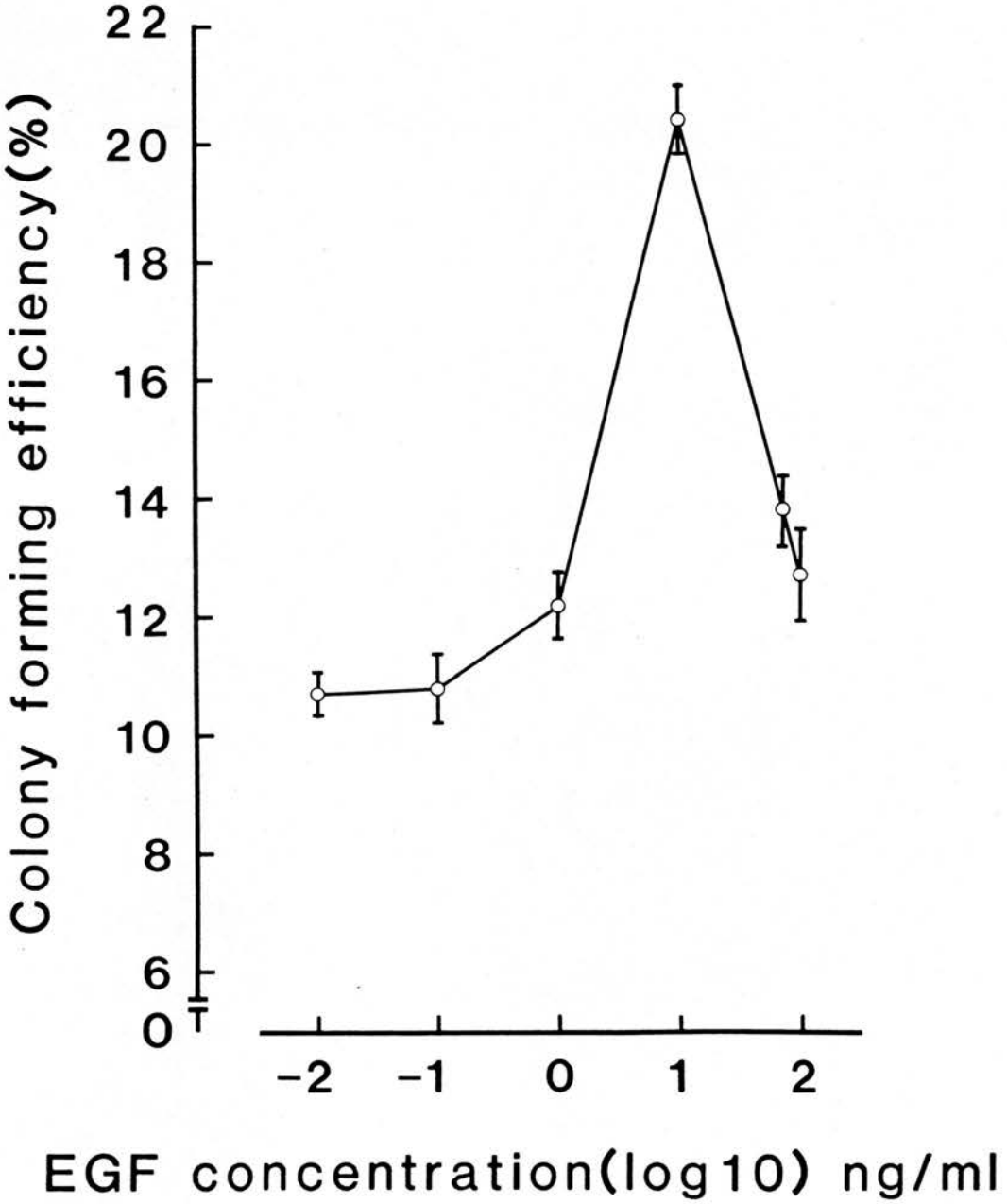
One hundred thousand JS7 cells (passage 98) were seeded in agar as described in "General Materials and Methods". EGF was incorporated at the indicated concentrations (0.01, 0.1, 1, 10, 50, and 100 ng/ml) in both the agar base and overlay medium. Colonies 50μm or more in diameter were scored after 14 days of growth and expressed as a cloning-efficiency. Values represent the means of two experiments, in each of which triplicate plates were used.

Results

Results shown in Fig.4.20 and Fig.4.21 indicate that there was a dose-dependent stimulation of colony formation by EGF. Compared to the untreated control cells, significant ($P < 0.001$) stimulation was demonstrated with as little as 0.01 ng/ml (5.6% untreated

Fig. 4.20

Effect of various EGF concentrations on SPA colony formation. Note 10 ng EGF per ml induced maximum stimulation. Reduction in the stimulatory effect is apparent at concentration of 50 ng/ml or greater.


Colony forming efficiency(%)
22
20
18
16
14
12
10
8
6
0
-2
-1
0
1
2
EGF concentration(log10) ng/ml

vs 10.4% treated), and maximum stimulation achieved at an EGF concentration of 10 ng/ml ($P < 0.001$). An increase in the size of colonies was shown at 10 ng/ml compared to the lower doses. However, although incorporation of 50 ng or 100 ng/ml decreased the number of colonies, these concentrations of EGF increased the size of individual colonies to the extent that some were observable with the unaided eye after a period of three weeks.

Experiment 3

Effect of serial passage on cloning efficiency of JS7 cells

Introduction

It has been reported that growth in soft agar often is acquired only after many *in vitro* passages (Marshall *et al*, 1977). In earlier studies with SPA cells (Table 4.4), it was found that anchorage-independence was not apparent before passage 16 and that the addition of EGF enhanced their growth in soft agar. Because JS7 had been passaged over 140 times, it was of particular interest to examine whether increasing *in vitro* passage influenced colony-forming efficiency and whether EGF could produce a stimulatory effect similar to that exerted on the cells at low passages.

Experimental design

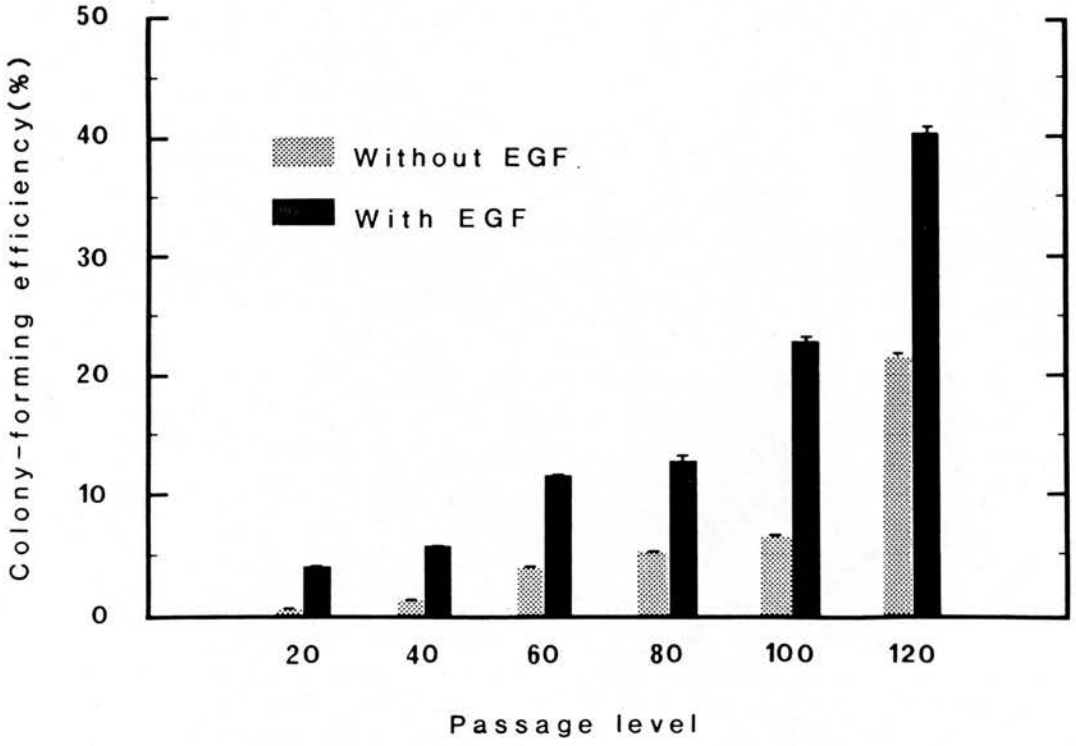
One hundred thousand cells of JS7, at various passage levels (20, 40, 60, 80, 100, 120), were plated in agar medium as described in " General Materials and Methods " in medium lacking or containing 10 ng/ml EGF. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Colonies were counted after 14 days incubation.

Results

Results in Fig. 4.21 indicate that increasing cell passage was associated with an increase in cloning efficiency, both with and without EGF. Again, at each passage level EGF enhanced the colony forming efficiency until at passage 120 this was 40%. The size of colonies produced by these cells increased with increasing cell passage. The increase was apparent both in the presence and absence of EGF, but colonies always were larger in the presence of EGF. At the end of 14 days, colonies of EGF were 2-3 times the size of untreated colonies. At every passage level tested, the difference between the size of colonies in the absence and presence of EGF became progressively larger. There was no difference in the size of cells forming the colonies.

Fig. 4.21

Effect of the increasing passage number on the cloning efficiency of JS7 cells.


50
40
30
20
10
0
Colony-forming efficiency(%)
Without EGF
With EGF
20
40
60
80
100
120
Passage level

Experiment 4

Effect of cell density on colony-forming efficiency

INTRODUCTION

It has been reported that colony formation is related to the number of cells plated. Thus, the colony forming efficiency is affected by the number of cells plated and a minimum number is required (Leavitt et al, 1977; Von Hoff et al,1980).

In the present experiment the relationship between the number of cells plated and the colony forming efficiency in the presence or absence of EGF concentration was studied.

Experimental design

JS7 cells (Passage 120) were plated at varying densities: 10^3, 5×10^3, 10^4, 5×10^4, 10^5 cells per plate, in medium containing or lacking 10 ng/ml EGF. Three plates were used for each of the cell densities. Colonies were scored after 14 days' incubation.

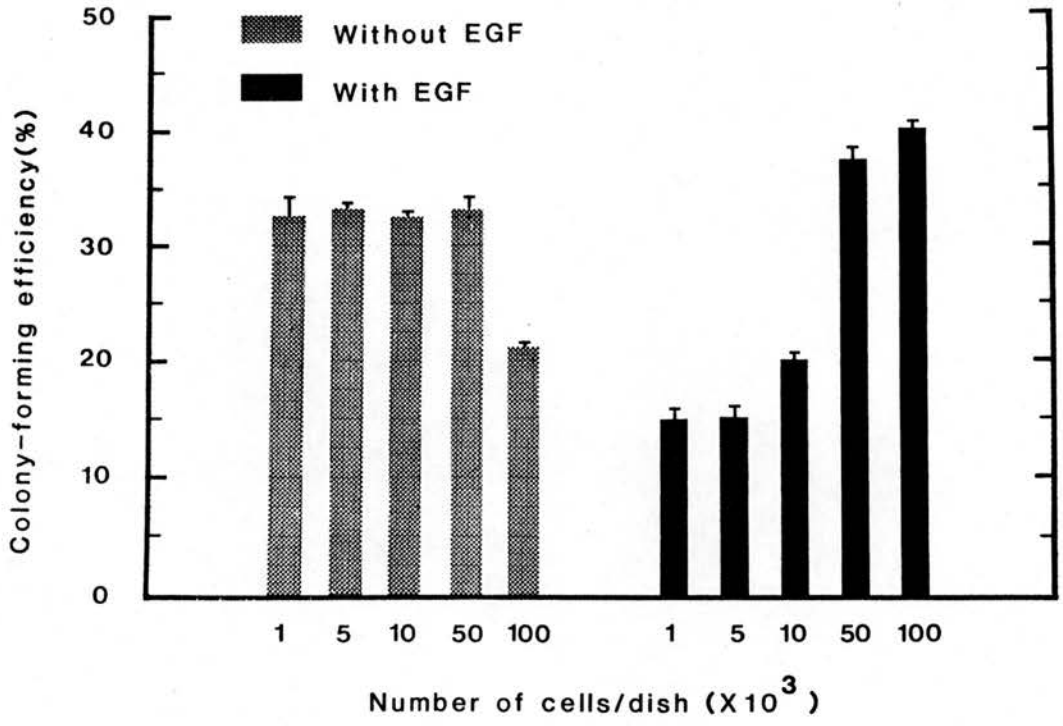
Results

Colony forming efficiency

As noted in Fig. 4.22, no significant difference in the cloning efficiency was determined when the cells were plated out in the range of 10^3 to 5×10^4 cells per plate. However, increasing cell density from 5×10^4 to 10^5 decreased the percentage of colonies formed.

Fig. 4.22

Effect of cell density on the colony forming-efficiency of JS7 cells.


Without EGF
With EGF
Colony-forming efficiency(%)
50
40
30
20
10
0
1
5
10
50
100
1
5
10
50
100
Number of cells/dish (X10^3)

In contrast, in the presence of EGF, cell densities of 10^4 or below, resulted in significantly ($P < 0.001$) lower cloning efficiency than in those without EGF. However, the cloning efficiency was increased by increasing the plating density to 5×10^4 and above.

Colony number

When the results of this experiment are presented in terms of cell numbers (Fig. 4.23) they indicate that increasing cell density increased the number of colonies formed per plate. In the presence of EGF, a linear relationship was demonstrated between the number of cells plated and the number of colonies found after 14 days of incubation. In contrast, the number of colonies formed in the absence of EGF declined between 5×10^4 and 10^5 cells.

Histologic characteristics of colonies

The appearance of colonies in soft agar in the absence or presence of EGF was somewhat similar to that seen in lung *in situ* or nude mouse tumour. Cells were relatively large with well-developed cytoplasmic boundaries and appeared uniformly ovoid or cuboid, displaying sizeable and prominent nucleoli (Fig. 4.24). Vacuoles were a constant feature in nearly all colonies.

Electron micoscopy

Findings of transmission electron microscopy of colonies were consistent with cells of epithelial

Fig. 4.23

Effect of increasing the number of cells plated per dish on the number of colonies of SPA cells per dish.

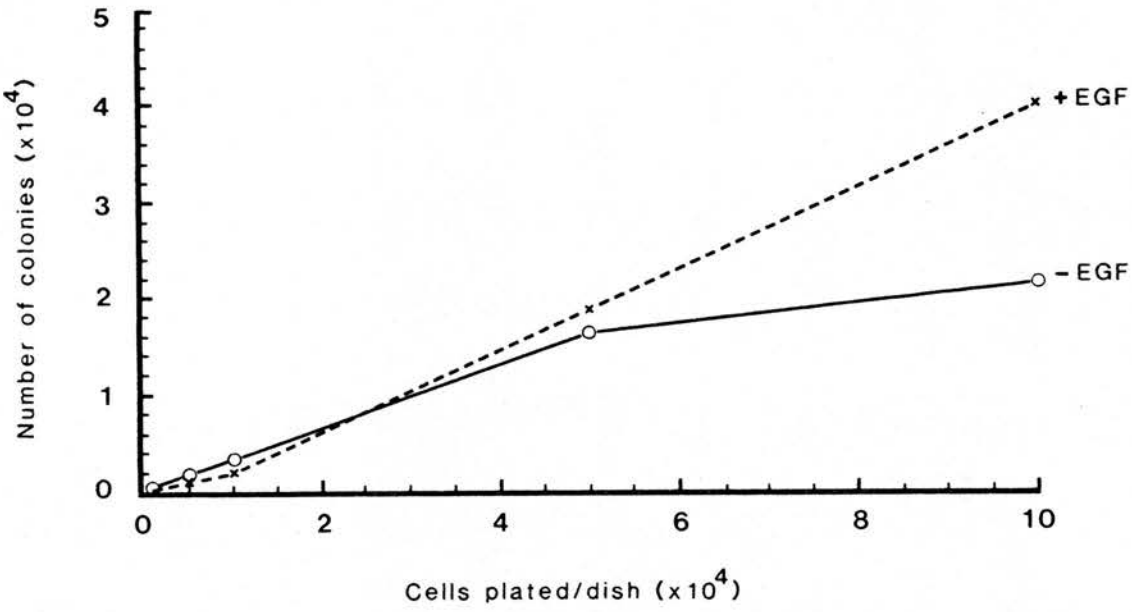
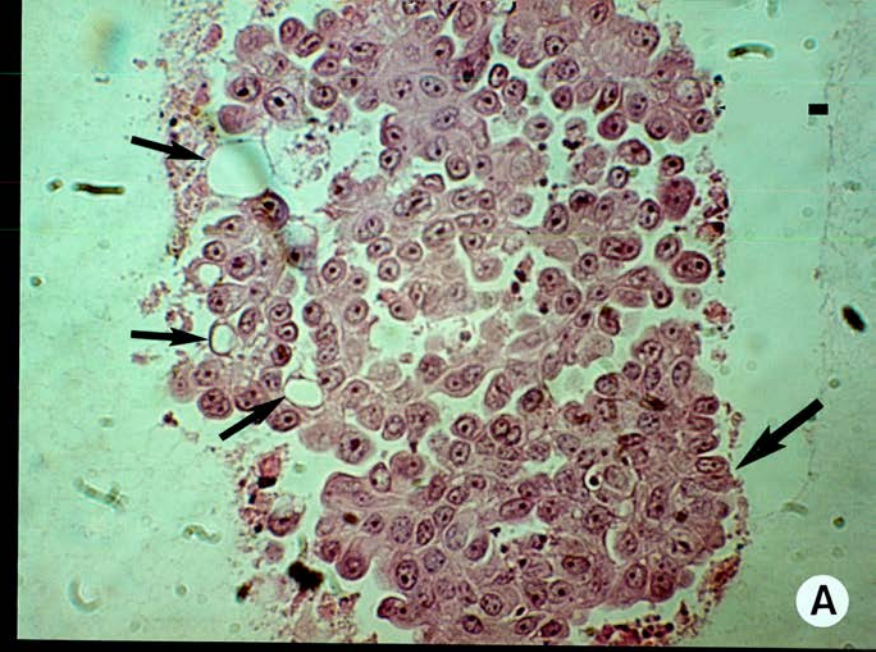
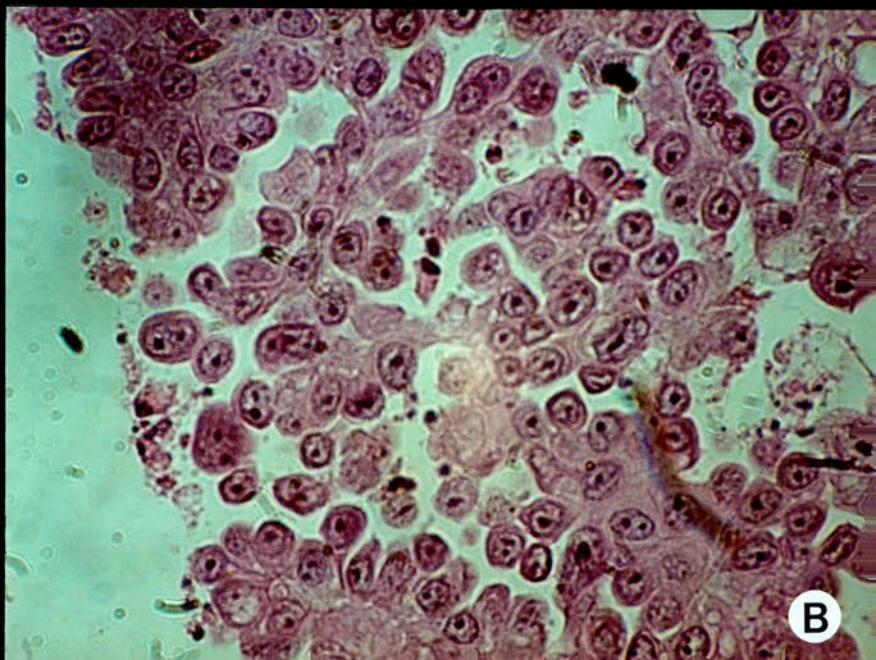

Number of colonies (x10^4)
5
4
3
2
1
0
0
2
4
6
8
10
Cells plated/dish (x10^4)
+EGF
-EGF

Fig. 4.24A,B

Histological section of SPA colony. A), Note cells forming structures similar to the papillary ingrowths in SPA lung *in situ* (large arrows) and vacuoles (small arrows).
B), same as in A but at higher power. Hematoxylin and eosin stain.
A, x 410; B, x 960.


A


B

origin. Cells appeared rounded and flattened or attenuated. Numerous microvilli covered the cell surface and desmosomes between cells were evident (Fig. 4.25B). Additionally, moderate folding and interdigitation of the nuclear membrane were occasionally observed. Mitotic figures were common (Fig. 4.25B).

Vesicles containing lamellated structures of various sizes were commonly seen (Fig. 4.25A). No glycogen deposits were seen in any of the colonies.

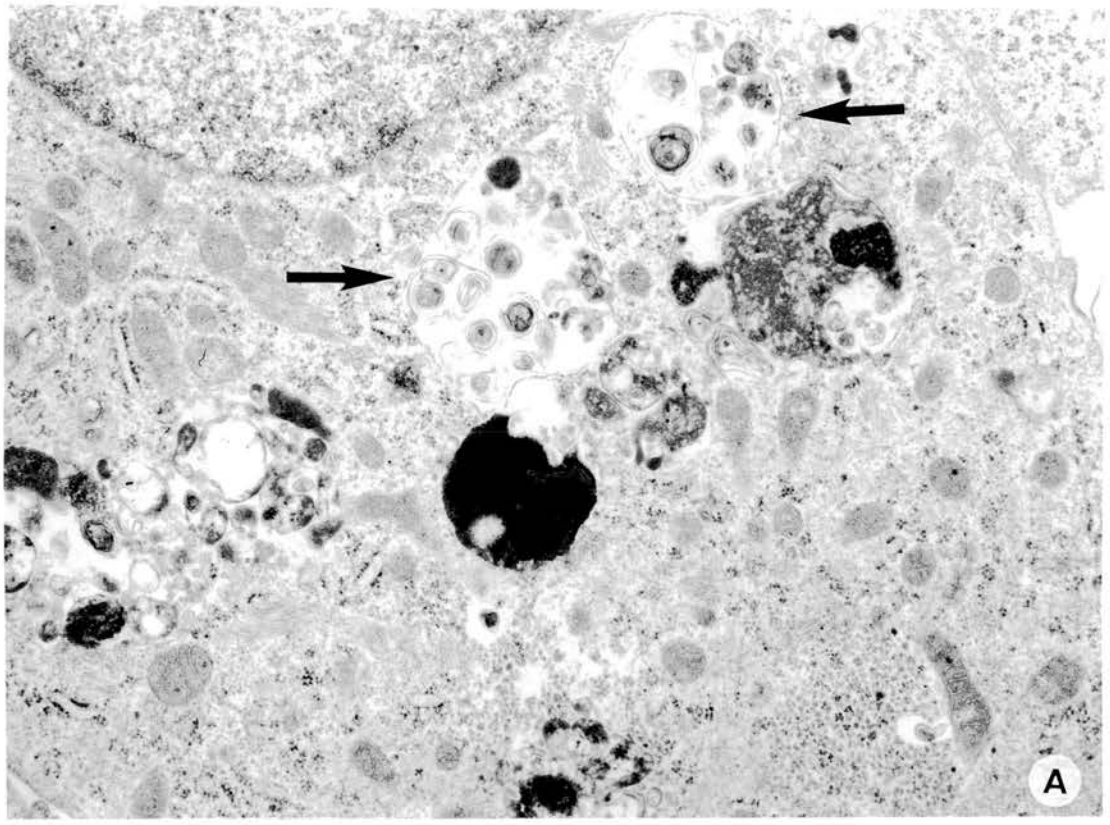
Recloning of colony cells

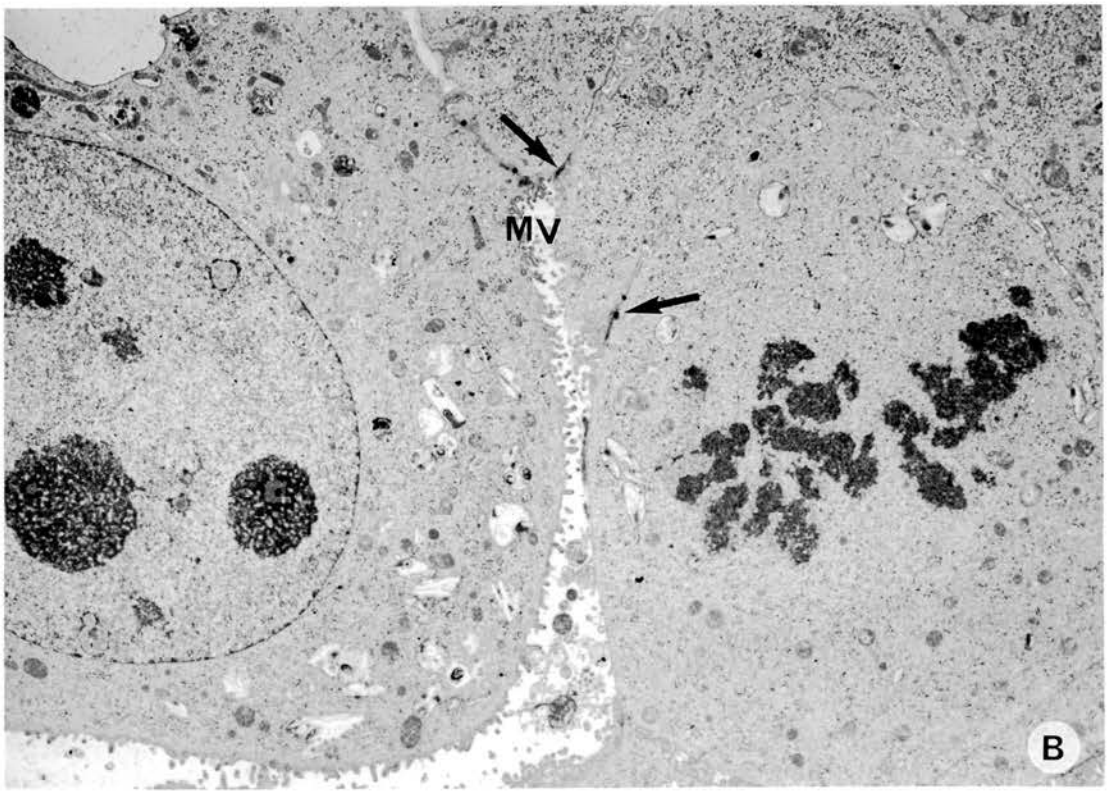
Cells of JS7 at passage 57 were plated in soft agar medium. After 24 hours' incubation, cells of colonies had begun to flatten out on the surface of the plastic underneath the agar layer. Cells formed nearly complete monolayers within 5-7 days. The clones maintained epithelial morphology indistinguishable from that of the parent cells. Some of these colonies were reassayed for growth in agar in the absence of EGF, and their cloning efficiencies varied from 5.6% to 18.5%.

Fig. 4.25A,B

Transmission electron micrograph of cells within SPA colony that had formed for 14 days in soft agar. B), Microvilli (MV) apparent on the cell surface and a number of desmosomes between cells (arrows) and mitotic figures can be identified in one cell.

A), a portion of cell showing vesicles containing lamellated structures (arrows). A, 4000; B, 20000.


A


MV
B

C). FUNCTIONAL CHARACTERISTICS

INTRODUCTION

In lung cell preparations, cells can be identified by their morphological and ultrastructural appearance. Among other criteria that can be used in the designation of a cell type are functional properties. In the case of type II pneumocytes a unique function is the synthesis and secretion of surfactant components, such as phosphatidylcholine (PC) and dipalmitaphosphatidylcholine (DPPC). In the present study, cell lines were initiated from SPA tumours that result most commonly from the transformation of type II pneumocytes (Perk et al, 1971). Ultrastructural examination of the initiated cell lines revealed lamellar bodies in some and none in others. Therefore, an attempt to determine whether these cells retained functional properties of type II cells was undertaken by measuring PC and DPPC synthesis and secretion.

Materials and Methods

Culture medium

The medium used was F12K medium supplemented as described in "General Materials and Methods."

Cell lines tested

Cell lines used in this study were JS7 passage 39, JS8 passage 22, and JS14 passage 11.

Experimental design

Triplicate cultures of each cell line were plated at a density of 2×10^5 cells in 25 cm^2 plastic flasks and incubated at 37°C. Cells were allowed to grow to near confluence or complete monolayers. At this time the medium was discarded and fresh medium (4 ml/flask) containing 1µCi of [^{14}C]-choline chloride (specific activity 58m Ci/mmol.) was added. Cultures were incubated for 24 hours at 37°C. After this time, the medium of one culture of each cell line was removed, the cells spun out and the medium stored at -20°C until analysed. The cell monolayer was washed twice with 3 ml of 0.85% (0.15M) sodium chloride solution. Thereafter, both the washed cells and the collected culture medium were frozen at -20°C. This same procedure was repeated at 3 and 5 days post confluence. Controls for this experiment comprised two samples of medium without any cells but containing [^{14}C]-choline chloride incubated for 24 hours at 37°C. At this stage the frozen samples were packed in solid CO_2 and sent to Dr R.J.Richards of the Department of Biochemistry, University College, Cardiff, for PC and DPPC assay.

RESULTS

The results obtained indicated that the use of radioactive material had no effect on the morphology of cells.

The PC and DPPC content of SPA cell lines is shown in Table 4.5. PC and DPPC were found in each of the cell lines at day 0 (confluence) at concentrations varying from 9.3-15% for PC and 0.4-3.3% for DPPC. The amount of PC and DPPC on day 3 of confluence was 7.7-16.1% for PC and 1.4-2% for DPPC. However, in cells tested after 5 days of reaching confluency, the amount of PC and DPPC was between 3.3-28% for PC and 0.4-0.8% for DPPC. Cells showed no potential to secrete PC and DPPC in culture medium.

Table 4.5

Phosphatidylecholine content of SPA cells

Samples	Label in PC (%)		Label in DPPC (%)	
	Cells	Medium	Cells	Medium
JS7				
Confluent	15.0	0.1	3.3	-
3 days	16.1	0.25	2.0	-
5 days	3.3	0.2	0.8	0.03
JS8				
Confluent	10.5	0.4	2.6	0.07
3 days	7.8	0.2	1.4	0.03
5 days	28.0	0.6	9.1	0.10
JS14				
Confluent	9.3	0.09	0.4	-
3 days	7.7	0.1	0.8	-
5 days	10.1	0.1	0.4	-
Controls				
Confluent		0.1		-
3 days		0.05		-
5 days		0.05		-

D). RETROVIRUS ANTIGEN DETECTION IN SPA CELLS

INTRODUCTION

Replication of the SPA retrovirus has been demonstrated in cell cultures initiated from tumours induced experimentally in young lambs by the intratracheal inoculation of SPA lung fluids (Sharp et al, 1985).

Although the application of immunobotting for detecting SPA-associated P25 antigen has greatly expanded our understanding of the relatedness of SPARV to other retroviruses (Sharp and Herring, 1983; Sharp et al, 1983), such a technique is cumbersome. Therefore, an easy assay system, such as the cocultivation technique, would be advantageous.

Cocultivation of human and animal tumour cells in culture with indicator cell lines has been used as a means for the isolation and/or detection of retroviruses.

Jensen et al (1970) isolated a retrovirus designated Mason-Pfizer monkey virus (MPMV) by cocultivating tumour tissue of mammary carcinoma of a Rhesus monkey with monkey embryonic cell cultures. The virus did not cause visible alterations in the cocultivated cultures of monkey cells, and the culture continued to replicate the virus for at least 105 days post infection.

Virus-producing human cell lines were obtained by cocultivation of human embryonic cells with cells from breast carcinoma in a human patient. Production and

release of the virus with biochemical and biophysical properties of oncornavirus continued for up to 11 months of continuous *in vitro* passage (Keydar *et al*, 1973). Further viruses were isolated following cocultivation of human leukaemic bone marrow with nonproducer cells containing murine sarcoma (Nooter *et al*, 1977 & 1978). Since the discovery of XC cell fusion by Klement *et al* (1969), several strains of animal retroviruses have been found to induce syncytia in certain indicator cells cocultivated with virus producing cells (Weiss *et al*, 1982). Syncytial assays have been described for simian sarcoma-associated virus (Rangan *et al*, 1972), feline leukaemia virus (Rangan *et al*, 1973), Mason-Pfizer monkey virus (Rand *et al*, 1974), feline and baboon endogenous viruses (Rand and long, 1973; Ahmed *et al*, 1975; Tanaka *et al*, 1981) and bovine leukaemia virus (Diglio and Ferrer, 1976).

This study was undertaken to detect virus replication in the various SPA cell lines initiated from natural cases of SPA tumour, to initiate productive cell lines and also to assay them for the SPA retrovirus.

MATERIALS AND METHODS

Detection of SPARV

In an attempt to detect virus replication in culture, supernates of SPA cultures were collected at various intervals and processed for immunoblotting as described in "General Materials and Methods."

Cocultivation

Attempts to establish persistently infected cell lines were made by cocultivating SPA cultures which demonstrated SPARV-specific antigen with NBL12 (bat lung) and HTO1 (Tahr; a permanent cell line of Himalayan mountain goat ovary cells). For cocultivation, primary cultures of each of the four cell lines were mixed in suspension at a ratio of 1:1 with NBL12 or HTO1 cells and seeded on to 75 cm^2 plastic tissue culture flasks. Cocultivated cultures were passaged 10-20 times during which culture fluids were examined at various intervals for the presence of virus. For syncytial induction, SPA cells were cocultivated with XC cells at a ratio (SPA:XC) of 1:1, 1:2, 1:3 and incubated for one week. Cocultivated cultures were then stained with Giemsa stain and examined for syncytial formation.

RESULTS

The SPA retrovirus was detected in supernates of the four cell lines by immunoblotting (Fig. 4.26). The P25 antigen of SPA retrovirus was demonstrated in cultures of JS14, JS8, JS15, and JS7 for up to 19, 64, 70, and 176 days respectively (Table 4.6).

When cell lines were tested at passage levels beyond those indicated in Table 4.6, no indication of virus replication was demonstrated.

Assay for virus in cocultivated cultures

Blotting assay of co-cultivated SPA cells with NBL12 or HTO cells, demonstrated SPARV P25 in these cultures at passage levels similar to those of the non-co-cultivated cultures. However, when SPARV disappeared from the original SPA cell culture, the assay also failed to demonstrate the presence of virus replication in cocultivated cultures, despite several *in vitro* passages.

No syncytial formation was seen in any of the cultures cocultivated with XC cells.

Fig. 4.26

Immunoblots of SPA culture supernates.

Lane 1 : lung fluid from a natural case of SPA (JS53)

Lane 2,3 : supernate from culture of JS15 (passage 2)

Lane 4,5 : supernate from culture of JS15 (primary culture)

Lane 6 : supernate from culture of JS7 (passage 8)

Lane 7 : supernate from culture of cocultivated JS15/NBL12 (passage 1)

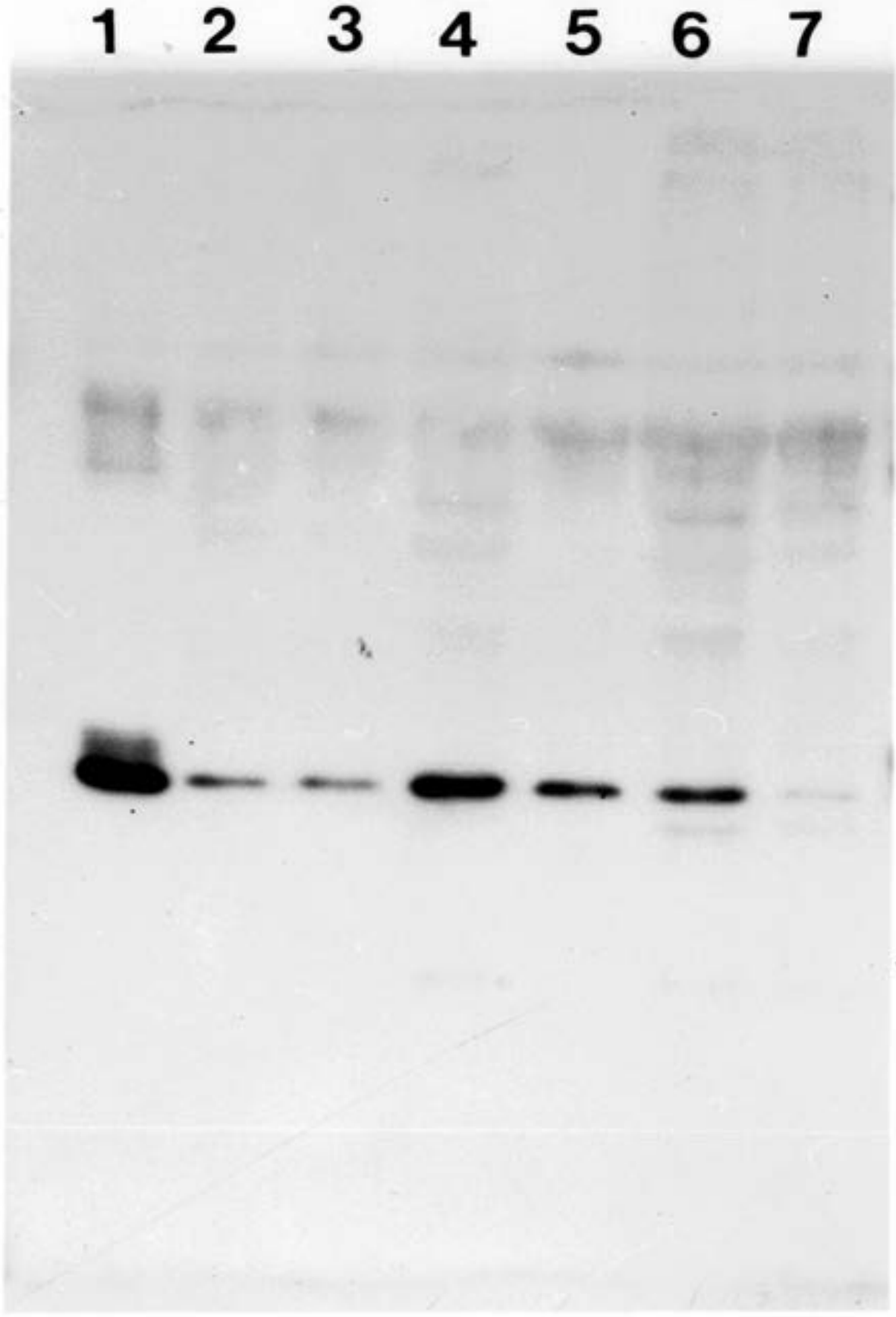

1
2
3
4
5
6
7

Table 4.6

Detection of P25 antigen of SPA retrovirus in cultures of SPA tumours

Cell line	Passage(s) at which P25 was detected	Days after trypsinisation when P25 last tested
JS7	5, 6, 7, 8, 9, 10, 11	176
JS8	1°, 1, 2, 3	64
JS14	1°	19
JS15	1°, 1, 2, 3, 4	70

1°= primary culture.

DISCUSSION

In this chapter are described the establishment of continuous lines of SPA cells and some of their characteristics. The fact that two of the cell lines, JS7 and JS8, have been maintained serially *in vitro* over three years for more than 140 passages, is much longer than is usually achieved with normal cells (Hayflick, 1965). Prior to this, no permanent line of cell cultures was available from SPA.

A review of the literature suggests that the only report of the establishment of an epithelial cell line from a SPA tumour is that by Coetzee *et al* (1976). However, it is interesting to note that when Coetzee's cell line was examined in this laboratory it failed to grow beyoned 37 passes, which rather suggests that it lacks the potential to grow continuously in culture. For this reason the results of this present study indicate that JS7 and JS8 are, to date, the only permanently established cell lines derived from a SPA tumour.

Several features support the previous morphological observations that the SPA cells are epithelial. Indeed, the fact that the cells comprising each of the SPA cell lines are epithelial is demonstrated by their ultrastructural characteristics. These included the presence of numerous microvilli, clearly defined desmosomes in the area of cell-cell contact, and bundles of tonofilaments all of which have been described

previously as markers of epithelial cells (Owens et al, 1974) and have been demonstrated in a variety of epithelial cell lines (O'Toole et al, 1976; Engel et al, 1978; Hechman et al, 1978). In addition, functional properties such as the capacity to transport fluids in culture, as shown by the formation of hemicysts, are features consistent with a secretory epithelium.

Fetal type II pneumocytes are capable of storing large amounts of glycogen at early stages of gestation but the situation is completely reversed during late gestation and in adulthood (Kikkawa et al, 1968). The appearance of glycogen in transformed type II pneumocytes in SPA tumours is considered to be a phenomenon of retrodifferentiation (Perk et al, 1971). In the present study the proportion of cells containing glycogen was variable depending on the particular cell line. Glycogen was a common feature of JS7, but was present only in small quantities in JS8 and in none of the JS14 cells. However, several workers, who studied the ultrastructure of SPA lesions, reported conflicting results regarding the amount of glycogen seen in the tumour cells (Perk et al, 1971; Nisbet et al, 1971; Cutlip and Young, 1982; Payne and Verwoerd, 1984). For example, Nisbet et al (1971), and Payne and Verwoerd (1984), observed that only a small proportion of tumour cells contained glycogen, whereas, Perk et al (1971), and Cutlip and Young (1982) noted that many of their tumour cells contained large amounts of glycogen. These

observations suggest that the glycogen content, which varies from one cell to another, is most likely to be a specific feature not only of each cell line in culture but also of the original tumour. This hypothesis is supported further by the fact that glycogen in nude mouse tumours (Chapter 6) may be present or absent, as is the case in the respective tumour-derived cell lines.

Hemicysts were observed consistently in each of the four epithelial cell lines derived from SPA tumour reported in the present study and is, we believe, the first report of the formation of hemicysts by cells from SPA. Although no efforts had been made to encourage hemicyst formation by alteration of cultural conditions or to document their occurrence after numerous passages in culture, the hemicysts continued to be produced in lines JS7 and JS8 even after long-term in vitro cultivation for more than 140 passages. These observations suggest, therefore, that hemicyst formation can be regarded as an inherent property of these cells.

Although the significance of hemicyst formation has not been established, a number of workers consider it to be an unequivocal identifying feature of epithelial cells involved in fluid transport, or that it may represent a differentiated function of transporting epithelium (Soule et al, 1973; Pickett et al, 1975; Toyoshima et al, 1976; Lever, 1979). In this present work the hemicysts formed by SPA cells appeared similar to those found in other epithelial cultures (McGrath and

Blair, 1970; Owens et al, 1974; Pickett et al, 1975) and further studies showed that hemicyst formation results from fluid accumulation beneath the cell layer, between the basal surface of the cell and the solid substrate on which it is growing. The significance of the continued generation of these structures by SPA cells in vitro suggests the maintenance of some secretory functions by the epithelial SPA cells. This notion is supported both by the formation of cystic tumours in nude mice inoculated with SPA cell lines (Chapter 6) and the studies of Mason et al (1982) and Goodman and Crandall (1982), who reported hemicyst formation in primary cultures of alveolar type II cells derived from rat lungs. They concluded that hemicyst formation indicates an active transport function of the cells in culture, and may represent an important mechanism in the regulation of normal alveolar fluid balance in mammalian lungs in vivo.

Normal ovine cells have 54 chromosomes consisting of 3 pairs of large metacentric, 2 sex chromosomes, and 48 acrocentric or telocentric chromosomes (Hsu and Benirschke, 1967), but karyological analysis showed that the SPA cell lines had a hyperdiploid (JS7, JS8), diploid (JS14) or hypodiploid (JS15) modal number, and were also highly aneuploid. Thus, the interpretation of chromosome behaviour in SPA cells in vitro and the manner in which their constitution relates to that of the original tumour from which the cells were derived remains a problem.

Fraccaro et al (1969) studied the karyotype stability in long-term culture and found that normal karyotypes do not necessarily predispose to instability, and that long-term cultures may accurately reflect the tissue of origin. Other studies of cell lines showed little change in karyotype over a period of one year in culture (Moore and Sandburg, 1964).

Dexter et al (1978) found a similar chromosome complement in cell lines and in the tumour from which they were derived, and suggested that the genotypic heterogeneity observed in the derived cell lines had its origin in the neoplasm itself. Based on these reports, the aneuploid structures noted in the SPA cell lines may reflect the chromosome complement of the original tumour from which the cell lines were isolated. It is well known that the lungs of sheep with pulmonary adenomatosis show great variation in ultrastructural appearance even in a single tumour (Perk et al, 1971), and thus the distribution of chromosome numbers may reflect the morphological pleomorphism of the tumour.

One must also consider the possibility that the chromosome pattern obtained in this study may not reflect the actual chromosome constitution in the original tumour, since a numerical drift in chromosome complement has been reported in some cell lines resulting from continual propagation in vitro (Giraldo et al, 1971).

Dicentric or abnormally large chromosomes were found in JS7 and JS15 cell lines. The existence of these

abnormal chromosomes suggested that interchromosomal exchange had occurred during transformation. The appearance of unusual dicentric or large marker chromosomes has also been described in several human tumours and in cell lines derived from them (Tumilowics et al, 1970; Biedler et al, 1973; Miyaki et al, 1982). A marker chromosome with a secondary constriction was also found in cell lines derived from Burkitt's lyphomas and carrying the Epstein-Barr virus (Kohn et al, 1967; Miles and O'Neil, 1967) and also in normal peripheral human leukocytes transformed in vitro after exposure to irradiated Burkitt's cells (Henle et al, 1967). These large rearranged chromosomes were believed to play a role in the conservation of genetic material in hypodiploid cells (McFall et al, 1977). It has also been shown that in tumour cells containing less than 46 chromosomes, the genome contains more than the diploid amount of DNA (Atkin, 1962).

One of the most interesting observations was the high incidence of double-minute chromosomes (DMs) in the JS8 cell line, which were not observed in any of the other SPA lines. These structures were described first in human tumours by Spriggs et al (1962). Although the presence and possible derivation of these chromosomes has been discussed in great detail (Mark, 1967; Levan et al, 1968; Sandberg et al, 1972), their origin remains uncertain. Even though most frequently reported in tumours of neurogenic origin (Lubs and Salmon, 1965; Levan et al,

1968; Schlesinger et al, 1976), they also have been found in a variety of human tumours and murine sarcoma. White and Cox (1967), found double-minute chromosomes in 5 to 10% of mitoses of human rhabdomyosarcoma after eight months in culture. Their presence was also reported in many metaphases of human thyroid carcinoma cells examined at various stages of cell culture (Jones et al, 1967). Mark (1967), observed this type of chromosome in Rous sarcoma in mice. Double-minute chromosomes were also demonstrated in all or most of the cells in three human malignant gliomas and in five others sporadically (Mark and Granberg, 1970). They were also found in a cell line derived from human pancreatic adenocarcinoma (Kryiazis et al, 1983). Double-minute chromosomes were also found in some highly transplantable adenovirus transformed Syrian hamster cell lines (Nachtigal et al, 1971), but their relation, if any, to malignancy is unknown.

The observation concerning the morphology, and size of double-minute chromosomes in JS8 cells is reminiscent of that described by Biedler et al (1973), in a continuous cell line established from a human neuroblastoma tumour. In this study the authors indicated that the DMs were variable in size, and were classified as "barely visible", "faint" (as the most commonly observed), "small" and "medium".

The detection of DMs in the JS8 cells is of interest as these chromosomes are cytogenetic manifestations of amplified DNA (Gilbert, 1983; Little et al,

1983; Brodeur et al, 1984), and DMs have been implicated in amplification of the oncogene N-myc in neuroblastoma and retinoblastoma (Brodeur et al, 1984; Lee et al, 1984; Schwab et al, 1984). That only a fraction of the JS8 cells contained DMs is consistent with previous observations in primary tumours and established cell lines (Schwab et al, 1984).

The karyotypes in the 4 SPA cell lines described in the present work are in marked contrast to those found by Coetzee et al (1976) in their "15.4" cell line derived from a SPA tumour. Coetzee's cell line at a passage level similar to that used in the present study, contained 70 to 85% normal diploid chromosomes. In view of the reported variation of chromosomes in the cell lines in the present study, compared to cell line 15.4, the chromosome variability in our cell lines may be due to the greater heterogeneity of the cells in culture.

Growth of cells in soft agar, as exemplified by their ability to produce colonies, is considered as an expression of the transformed state.

At low passages, SPA cells failed to produce colonies in semisolid agar but, after further passage, they acquired the ability to produce colonies. This was detected first between 16-20 passages and their cloning efficiency increased with higher passage numbers. These observations were consistent with the previously published work that growth in agar is a criterion of transformation acquired late after many in vitro passages. The studies of

Marchok et al (1978) with epithelial cell lines isolated from tracheal transplants preexposed to the carcinogen dimethylbenz(a)anthracene, showed that the efficiency of colony formation in agar of these cells increased or was often only acquired with higher passage numbers and may be a feature of late stages of transformation. Similar observations were reported by Fusenig and Dzarlieva (1982) in a mouse keratinocyte cell line (HEL).

In the present study colonies were examined histologically, and it was gratifying to observe that important characteristics of the SPA tumour were similar for tumour biopsies and cultured cells. Thus, the histological studies indicated that no major morphological changes to the structure of the cells had occurred during cultivation, while the formation of structures resembling papillary ingrowths supported by stroma-like material in colonies, demonstrated that the colonies had retained an important histological characteristic of SPA tumour. The derivation of the stroma-like material is unknown, however one can speculate that it may represent a cellular matrix.

Transmission electron microscopy of colonies revealed cells with prominent surface microvilli and desmosomes. These structures are consistent with the features demonstrated by SPA cells in monolayers or lung tissue in situ. However, JS7 cells under liquid culture conditions were well-differentiated, and contained lamellar bodies as well as glycogen granules. The lack of

lamellar bodies may be due to the vigorous integrity of cellular growth *in vitro*, since cells in their actively growing phase usually are much less functional in terms of secretion or production of particular substance. Alternatively, there is preferential growth of this cell type in agar.

EGF was used in the present study to examine its influence on the SPA cells. The results of experiment 4 to determine the effect of cell density on cloning efficiency indicated that the capacity of cells to form colonies in soft agar in the absence of EGF was much affected by the density at which the cells were plated. Increasing cell density from 5×10^4 or below to 10^5 decreased the percentage of colonies formed. The reducion in the efficiency of colony formation with cell densities greater than 5×10^4 may have resulted from the depletion of growth factors contained in the fetal calf serum as has been reported for other cell types growing in agar (Leavitt *et al*, 1977; Von Hoff *et al*, 1980). In contrast, cells grown in the presence of EGF showed no reduction in cloning efficiency when cells were plated at 5×10^4 or greater and the linear relationship was maintained. This effect may have been due to replacement by EGF of the purported growth factor(s) in fetal calf serum. The above view is supported by the observations that EGF stimulation is independent of calf serum and that low concentrations of EGF stimulate cell growth when serum is added at low

concentration (Stoker et al, 1976; Osborne et al, 1980). Alternative explanations are possible to account for these observations. Although EGF stimulates replication of many cell types, it also has been reported to inhibit the growth of a variety of cell types that contain large numbers of EGF-receptors (Johnson et al, 1980; Gill and Lazar, 1981; Imai et al, 1982; Kamata et al, 1986). Furthermore, the sensitivity to this inhibitory effect of EGF correlated with the amount of EGF receptors (Kamata et al, 1986). Consequently, in the present study, the suppressive effect of EGF on the growth of SPA cells at low cell density may have resulted from overexpression of EGF receptors or saturating concentraion of EGF in relation to the number of EGF receptors per cell. Further experiments would have to be done to support this view.

The response of cells to EGF can be influenced by cell density, but the experiments in this area have produced conflicting results. For example Gill and Lazar (1981), found that increasing cell density resulted in an increased number of EGF receptors per cell and a concomitant inhibition. Similar results were obtained with Balb/c-3T3 cells (Pratt and Pastan, 1978) but the opposite occurred with BSC-1 cells (Holley et al, 1977).

With JS7 cells, EGF continued to stimulate growth when the cell density was increased. By extrapolation from the above studies with other cells, this continued growth may have resulted from a reduction in the number of EGF receptors per cell. Further studies

supporting this view have been reported by Kamata et al (1986) who found that reduction in the number of EGF receptors allows the cell to escape from the EGF inhibitory effect.

The phosphatidylcholine content of the SPA cell lines was assessed to gain further insights into the nature of the cells containing or lacking the lamellar inclusions. Although the cells in culture grew with epithelial morphology and expressed several ultrastructural features of SPA tumour cells their functional activities appeared reduced. The amounts of PC and DPPC in the SPA cell lines and secreted into the culture medium were low compared to freshly isolated type II pneumocytes from adult animals (Adamson and Bowden, 1974; Mason et al, 1977b) and declined to marginal levels the longer the cells remained in culture. Several cell lines of type II origin have been derived from lung tumours and, typically, the phosphatidylcholine content varies from 40 to 50% of the total phospholipids (Sanders, 1982). For example, the A549 cell, which is of human origin, contained only 41% phosphatidylcholine (Rooney et al, 1977), and cultured cells derived from human fetal lung contained 37% (Milo et al, 1984). Therefore, although JS7 cells exhibited lamellar bodies the phosphatidylcholine content of these cells is abnormally low when compared to the previous published values.

A small proportion of JS8 cells stained positively with Phosphine 3R. Although none of these cells were found to contain lamellar bodies, they still were capable of secreting phosphatidylcholine at higher levels than JS7 cells which contained lamellar bodies. This behaviour was not unique, because when A549 cells, in the present study, were stained with Phosphine 3R only 2% of the cells were positive (data not shown). Furthermore, Mason and Williams (1977) examined the A549 cells by transmission electron microscopy and found that some of these cells contained lamellar inclusion bodies. However, many of these structures appeared to be condensed autophagic vacuoles, yet the phosphatidylcholine content of these cells accounted for 54% of the total phospholipids. At this stage of the study no final conclusions can be reached concerning the cell types represented in the SPA cell lines. It is generally observed that the loss of tissue-specific properties occurs upon explantation of cells in vitro (Holtzer et al, 1960; Hilfer, 1962; Whittaker, 1963; Sato et al, 1960; Lambiotte et al, 1973; Auersperg and Finnegan, 1974), and the maintenance or the expression of functional properties of cells in vitro is highly dependent on the cultural conditions and on the nutrient environment (Deluca, 1966; Spooner and Hilfer, 1971; Lambiotte et al, 1973; Auersperg and Finnegan, 1974). In addition, there are several reports which demonstrate enhanced differentiation of poorly differentiated carcinoma when they were explanted

into culture conditions (Flaxman, 1972; Auersperg and Erber, 1976; Scarpelli and Rao, 1979). The culture medium used in the present study was Ham's F12K, which was originally modified for the cultivation of human liver cells and is considered to be one of the enriched media (Kaighn, 1973). It has been shown that for culture of normal type II pneumocytes, Ham's F12K medium supported a higher level of differentiation in these cells (Douglas et al, 1976). It is also possible that, in our cultures of SPA cells, some additional factors such as hormones and/or other nutrient components may be necessary for the expression and maintenance of the SPA cell function.

In this regard, it is extremely interesting to note that when cultures of SPA cells that contained no lamellar structures were exposed to bromhexine HCl, all cells demonstrated lamellar bodies when examined by phosphine 3R or by electron microscopy (Chapter 5). Additionally, the SPA cultures upon transplantation into nude mice, demonstrated secretory activities as evidenced by inducing fluid-filled cystic tumours (Chaper 6). It is reasonable to assume that host nutritional and/or hormonal factors present in the nude mice favoured the resurgence of the functional activities of cultured cells. It was suggested that the capacity to differentiate beyond the level attained in vitro appears to depend on the environmental factors present in culture, such as the presence of solid-liquid interface in culture (Auersperg and Erber, 1976). Another possibility is that the

development of connective tissue stroma around the tumour growing in the nude mice may facilitate enhanced differentation of tumour cells, analogous to the effects of mesenchyme on pancreas rudiment (Golosow and Grobstein, 1962; Rutter et al, 1978). This is supported by the observations of Terzaghi and Klein-Szanto (1980) who found that primary epithelial cell cultures derived from normal trachea of rat, required cocultivation with tracheal fibroblasts for growth and differentiation in subcutaneous Dacron pouches, and it was suggested that tracheal fibroblasts secrete factors necessary for normal tracheal epithelial cell growth and differentiation. It is known that fibroblasts secrete type-I as well as type-III collagens (Hance and Crystal, 1976). In addition, other studies showed that when fetal rats were injected intraperitoneally on day 17 of gestation, with a polypeptide stimulator, termed fibroblast pneumocyte factor, 3 days later their lung phospholipids showed enrichment in phosphatidylcholine (Smith, 1979). Other studies also showed that conditioned medium from fetal lung fibroblast monolayers evoked a significant stimulation of disaturated phosphatidylcholine (DSPC) in undifferentiated type II alveolar cells (Scott et al, 1983). In addition, the undifferentiated type II alveolar cells when grown in fetal bovine-supplemented medium showed the highest growth rate but the lowest level of disaturated phosphatidylcholine. Conversely, cells in

serum-free medium showed the lowest rate of the growth yet a rapid increase in radioactive precursor incorporation (Scott, 1987). Extracellular matrix (ECM) that contains different collagen types, proteoglycans, and glycoproteins has been found to play a significant role in sustaining growth and expression of differentiation in differentiated tissue (Li et al, 1987). The influence of ECM on cell morphology and differentiation appears to be modulated by receiving and integrating structural and functional signals that can direct specific gene expression in differentiated tissue. This may explain, in part, the failure to detect high phosphatidylcholine in SPA culture, since this study did not consider these variables. However, the precise mechanism of controlling factors influencing the state of differentiation of cancer cells is not yet known.

In the present study, replication of a retrovirus has been detected in cultures of each of the four cell lines. These findings not only confirm but also extend the observations of Sharp et al (1985) who reported virus replication in cultured cells derived from the lungs of lambs with experimentally induced jaagsiekte.

Virus replication was detected in early passes of the SPA cultures (Table 4.6), which thereafter, eventually disappeared from the culture. There is no clear explanation for the lack of virus replication although various interpretations could be put forward that might explain this perplexing situation. First, hemicyst

formation in SPA culture started to appear with increasing culture passages *in vitro*. Evidence has been obtained which indicates that exogenous RNA-dependent-DNA polymerase (RDDP) activity of all animal retrovirus strains examined so far, when grown in hemicyst-forming cultures, is low or inhibited. The inhibition of this essential enzyme activity is probably due to the production of a factor that is very effective in the inhibition of the exogenous RDDP activity (Lower *et al*, 1984). In addition Sharp *et al* (1983) were also unable to detect RDDP activity in SPA tumour explanted *in vitro*. Therefore, the ability of SPA cells to produce this factor which might have inhibited virus replication cannot be excluded. Secondly, a specific stage of differentiation may have a major role in regulating virus production by cells *in vivo* as has been reported for other cell systems (Lower *et al*, 1984). Such cells, when placed in culture, may not differentiate to a stage that permits virus replication.

SPA cultures were cocultivated with NBL12 and HTO1 cell lines in an attempt to induce a persistently infected cell line. No signs of persistent virus production were observed in any of the cocultivated cultures, when tested by immuno-blotting or examined by electron microscopy, but rescue of endogenous retroviruses by cocultivation may need months of intimate cocultivation (Todaro *et al*, 1978). SPA cultures were repeatedly cocultivated with the same batch of indicator cells and

passaged, in some instances, 20 times *in vitro* for a period exceeding 10 months. This may indicate that the indicator cells are non-permissive to SPA retrovirus or that a further period of cultivation is required. However, attempts by other workers to rescue virus from SPA cultures by cocultivation with indicator cell lines have also ended with negative results (Sharp, personal communication. Irving *et al*, 1984; Payne *et al*, 1986).

CONCLUSION

1. The SPA cell lines grown *in vitro* retain some features consistent with those of the SPA cells *in vivo.*
2. The persistence of the integrity of the structural and growth characteristics of JS7 and JS8 in culture indicates that they represent a suitable vehicle for biological studies of this type of tumour. Also the lines provide a means of propagating the cells beyond normal senescence and of undertaking studies, which require multiple passages of these cells, such as employing various agents to try and effect the expression of the virus in these cells. Moreover, the JS7 and JS8 cell lines will provide opportunities to examine different levels of differentiation properties in these transformed cells.

CHAPTER 5

REACTION OF SPA CELL LINES TO PREDNISOLONE AND BROMHEXINE HYDROCHLORIDE

INTRODUCTION

Lung surfactant is phospholipid-rich material that coats the alveolar surface. It decreases surface tension at the epithelium-interface and not only prevents the alveoli from collapsing, but allows uniform alveolar expansion (King and Clements, 1972; Goerke, 1974). The major phospholipid in surfactant is disaturated phosphatidyl-choline (DSPC) which is synthesized, stored and secreted by type II pneumocytes (Kikkawa et al, 1975; Massaro, 1981; Rooney, 1985). Morphologically, the surfactant appears as lamellar bodies in the type II pneumocytes (Askin and Kuhn, 1971; Batenburg, 1980).

A number of investigators have shown that glucocorticoids accelerate fetal lung maturation and stimulate surfactant production both in vivo and in vitro (Smith et al, 1973 & 1974; Farrell and Zachman, 1973; Khosla and Rooney, 1979; Khosla et al, 1980 & 1981). Cortisol-specific receptors have been found in fetal lung epithelial cells (Ballard and Ballard, 1974). Kotas and Avery (1971) reported that the fetuses of pregnant rabbits inoculated with prednisolone had more mature lungs than fetuses from uninoculated control rabbits. In addition to promoting surfactant production, prednisolone also strikingly alters the ultrastructural morphology of

cultured cells of mouse lung adenoma (Adamson and Klass, 1976). The prednisolone treated cells had more lamellar bodies per cell than did non-treated control cultures. These findings suggest that corticoids play a role in the maturation of lung epithelial cells.

Thyroid hormones also participate in the maturation of the lung (Hitchcock, 1979). They also appear to have some role in differentiation and phospholipid metabolism in type II epithelial cells (Redding et al, 1971). Oestrogen may also have a role in the maturation of fetal lung and the production of surfactant, as was shown by the administration of 17B-oestradiol to pregnant rabbits (Khosla et al, 1981). Oestrogen-treated fetuses demonstrated an increase in the percentage of differentiated type II alveolar cells compared with untreated controls. The hormone also increased the number of lamellar bodies per cell in treated groups compared with control groups.

Bromhexine HCl also influences fetal lung maturation. Exposure of human fetal lung cells in organ culture resulted in accelerated differentiation of type II pneumocytes as evidenced by the appearance of lamellar bodies (Bela and Angela, 1984).

The preceding characterization of JS7 and JS8 cells (Chapter 4) had led to the conclusion that JS7 cells exhibit several structural features commensurate with type II alveolar cells whereas further studies were required for the identification of JS8 cells.

Based upon the above review, JS7 and JS8 cell lines were exposed to prednisolone and bromhexine HCl to see if these two agents would enhance the expression of unexpressed features of these cells and thereby assist in identifying the nature of the cells, particularly that of JS8.

MATERIALS AND METHODS

Cells

Cells of JS7 (passage 120) and JS8 passage 108 were grown as described in Chapter 4.

Chemicals

Prednisolone stock solution was prepared by dissolving it in ethanol and diluting it with culture medium to give a final stock concentration of 1µg/µl. Bromhexine HCl was prepared by dissolving it in phosphate buffer saline at a concentration of 2µg/µl.

Plating efficiency

Approximately 500 cells were placed in each well of a 3 x 4 well tissue culture plate (Linbro, Flow Laboratories) in medium, and incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. One day later, the medium was changed: one group of three cultures, received standard medium, while the other groups of cultures were treated with medium containing the chemical (Tables 5.1 & 5.2). The cells were fed with fresh medium (2ml) containing the indicated concentrations of chemical every 48 hours.

After six days treatment cells were stained with buffered Giemsa as described in General Materials and Methods. Colonies of 8 cells or more were counted and the average colony formation expressed as the ratio of the total number of colonies in medium containing the chemical to that in the control medium.

Cell density

Cells (2×10^5) were plated in 25 cm^2 tissue culture flasks (Falcon, plastic) and grown at 37°C. After 24 hours, prednisolone and bromhexine HCl were added to the medium of 3 flasks to a final concentration of 20µg/ml, and the cultures incubated for a further six days. The medium containing chemicals was changed every two days. Finally, the cell density was calculated at the end of sixth day.

Electron microscopy

Ultrastructural investigation of the tissue culture material was performed on cell pellets obtained from treated and untreated cultures. The pellet was fixed in 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 and processed as described in General Materials and Methods.

RESULTS

Effect on cell morphology

Prolonged culture of SPA cells in the presence of prednisolone resulted in striking morphological changes (Fig. 5.1A). The cells lost their squamous epithelial shape and assumed a fusiform swirling appearance. Cells lost their granules, but maintained the ability to form hemicysts which became smaller in size and increased in number as the dose increased. After removal of prednisolone and addition of fresh culture medium to SPA culture, the cells reverted to the original epithelial morphology within 24 hours. The addition of bromhexine HCl to the culture medium resulted in a different pattern. The cells maintained their epithelial morphology, became smaller in size and contained numerous granules; even in JS8 cells which originally were without granules (Fig. 5.1B & C).

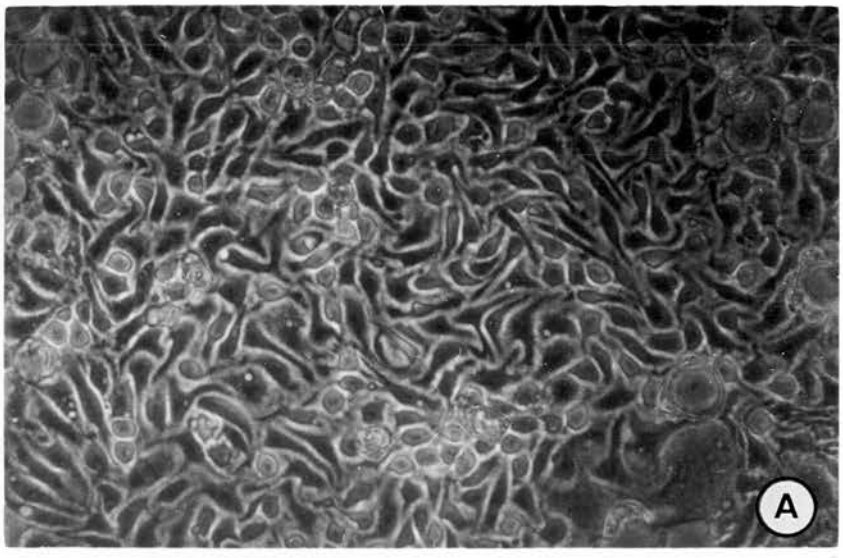
Effect on colony formation rate

From the results shown in Table 5.1 the growth of JS7 and JS8 cells appeared to be suppressed by prednisolone at concentrations of and above 0.08µg/ml and 1.25µg/ml respectively. Although colony formation rates of JS7 and JS8 cells decreased with increasing concentrations of prednisolone this was not significant ($P > 0.05$). Colony formation by JS7 cells was lower than that of JS8 cells at each concentration. Bromhexine HCl, however, had little effect on either cell line (Table 5.2).

Fig. 5.1A,B,C

A) JS7 cells treated for six days with prednisolone. Note the swirling appearance of the monolayers of cells. No intracytoplasmic granules are seen.

B) JS8 cells exposed to bromhexine HCl for six days. Note that the epithelial shape of the cells is maintained, but is more compact. x 205.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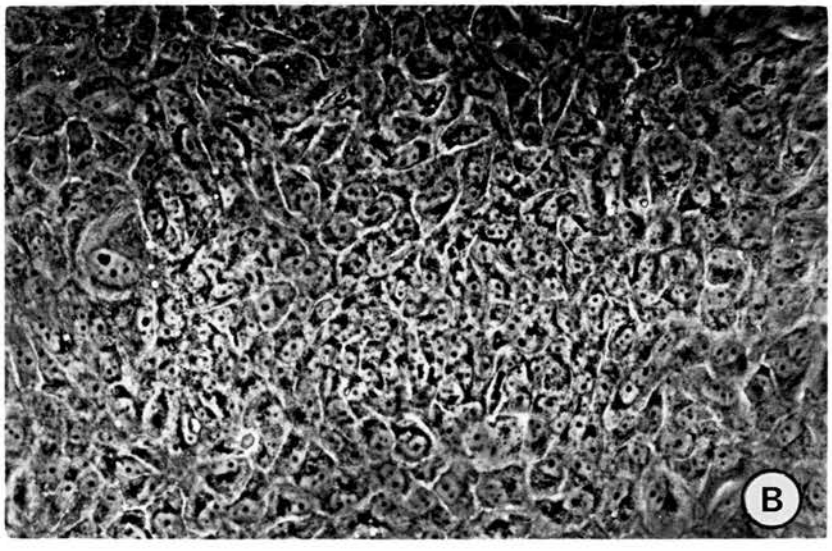

B

Fig. 5.1C

Cells of JS8 line treated for 24 hours with bromhexine HCl. Note the appearance of intracytoplasmic granules. x 550.

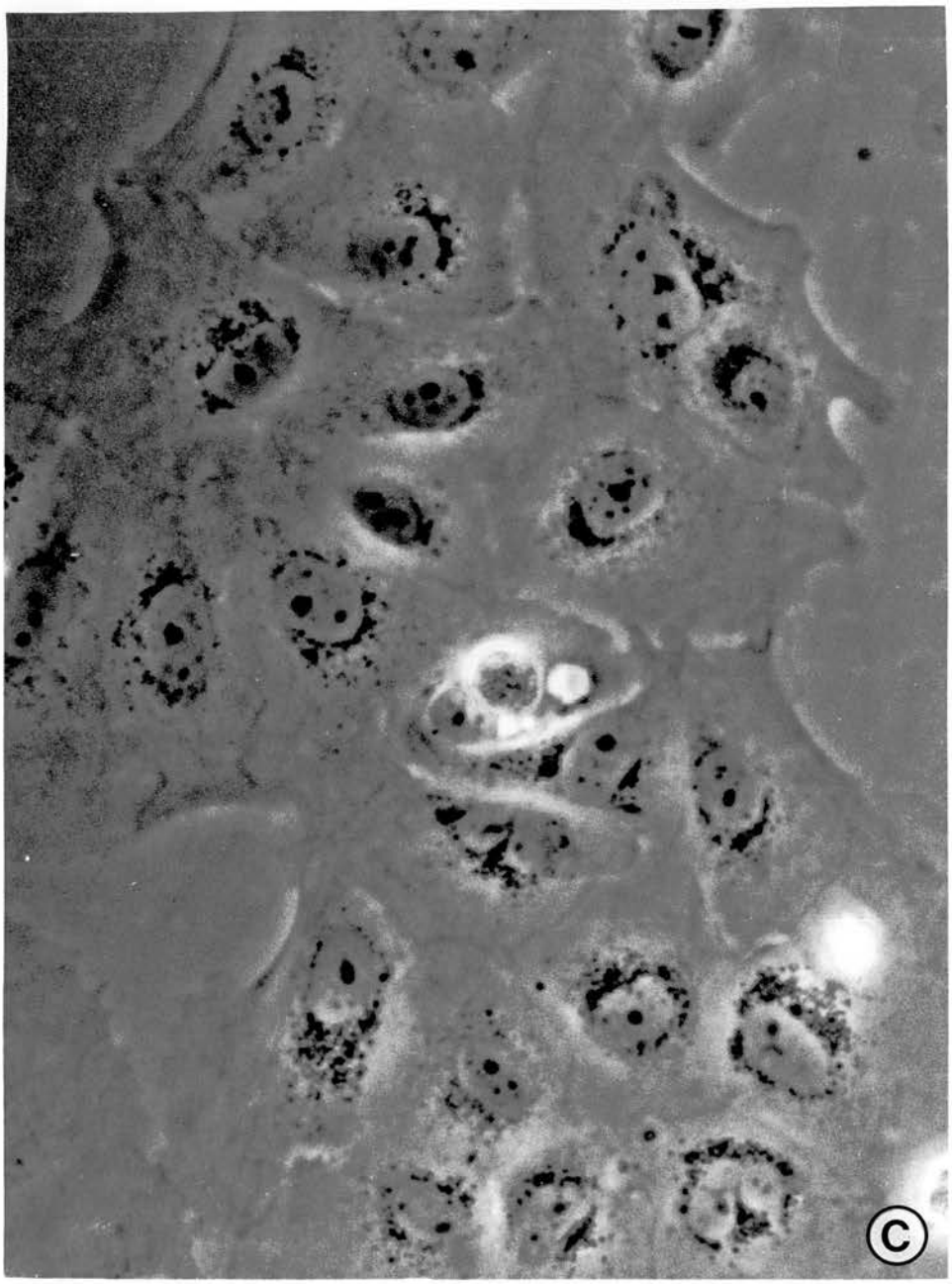


Table 5.1

Effect of prednisolone on colony formation rate of JS7 and JS8 cells.

Concent. of prednisolone	Number of colonies *		Colony formation rate,%	
(μg/ml)	JS7	JS8	JS7	JS8
0	252.7±4.70	207.3±5.70	100.0	100.0
0.02	254.0±4.62	208.3±5.24	100.5	100.5
0.08	246.7±4.37	207.7±5.17	97.6	100.2
0.31	241.3±4.18	207.7±5.61	95.5	100.2
1.25	240.0±4.16	203.3±5.46	95.0	98.1
5	237.3±4.10	200.3±5.49	93.9	96.6
20	235.7±4.41	200.7±5.36	93.3	96.8

* Values are means± SE.

Table 5.2

Effect of bromhexine HCl on colony formation rate of JS7 and JS8 cells.

Concent. of bromhexine HCl (μg/ml)	Number of colonies*		Colony formation rate, %	
	JS7	JS8	JS7	JS8
0	258.3±4.41	250.0±4.93	100.0	100.0
0.02	260.0±5.03	253.0±4.58	100.7	101.2
0.08	258.0±4.16	252.7±4.33	99.9	101.1
0.31	257.7±4.33	249.7±5.17	99.8	99.9
1.25	257.3±4.26	249.0±5.13	99.6	99.6
5	255.3±4.06	249.0±5.69	98.8	99.6
20	256.0±3.51	248.0±5.13	99.1	99.2

* Values are means± SE.

Effect on cell density

Cell density of JS7 cells decreased from 33.3×10^4 cells/cm² to 25.3×10^4 cells/cm² ($P < 0.05$) incubated with prednisolone at 20µg/ ml. Similarily, prednisolone reduced the cell density of JS8 cells from 33.6×10^4 cells/cm² to 24.7×10^4 cells/cm² ($P < 0.01$).

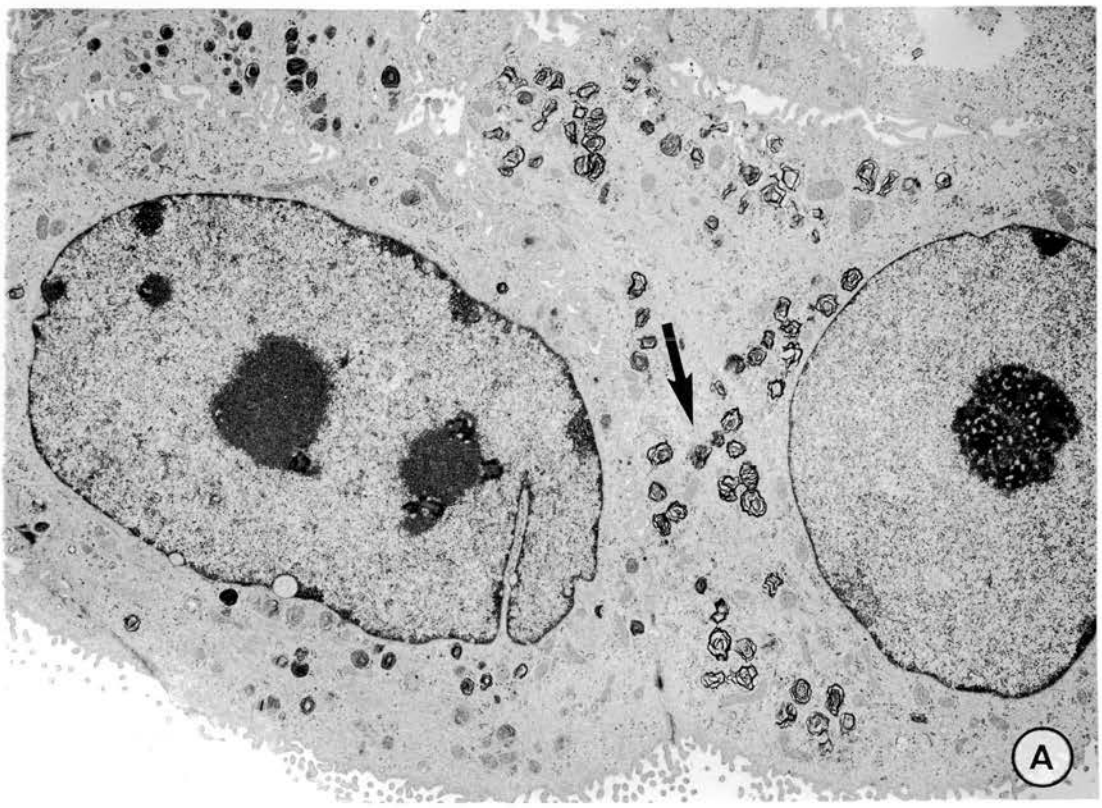
Bromhexine HCl slightly increased the cell density of both JS7 and JS8. These increased from 14.5×10^4 cells/cm² to 16.7×10^4cells/cm² in the case of JS7 and from 15.2×10^4 cells/cm² to 18.3×10^4 cells/cm² ($P < 0.01$) in the case of JS8.

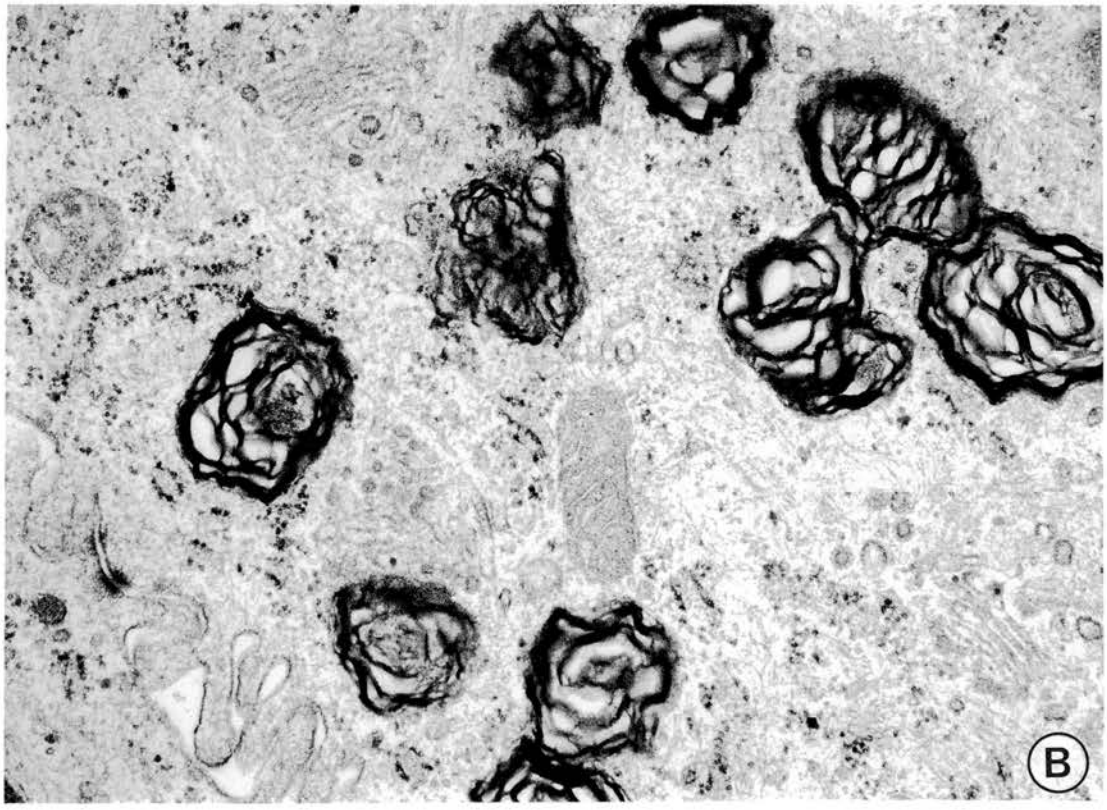
Ultrastructure

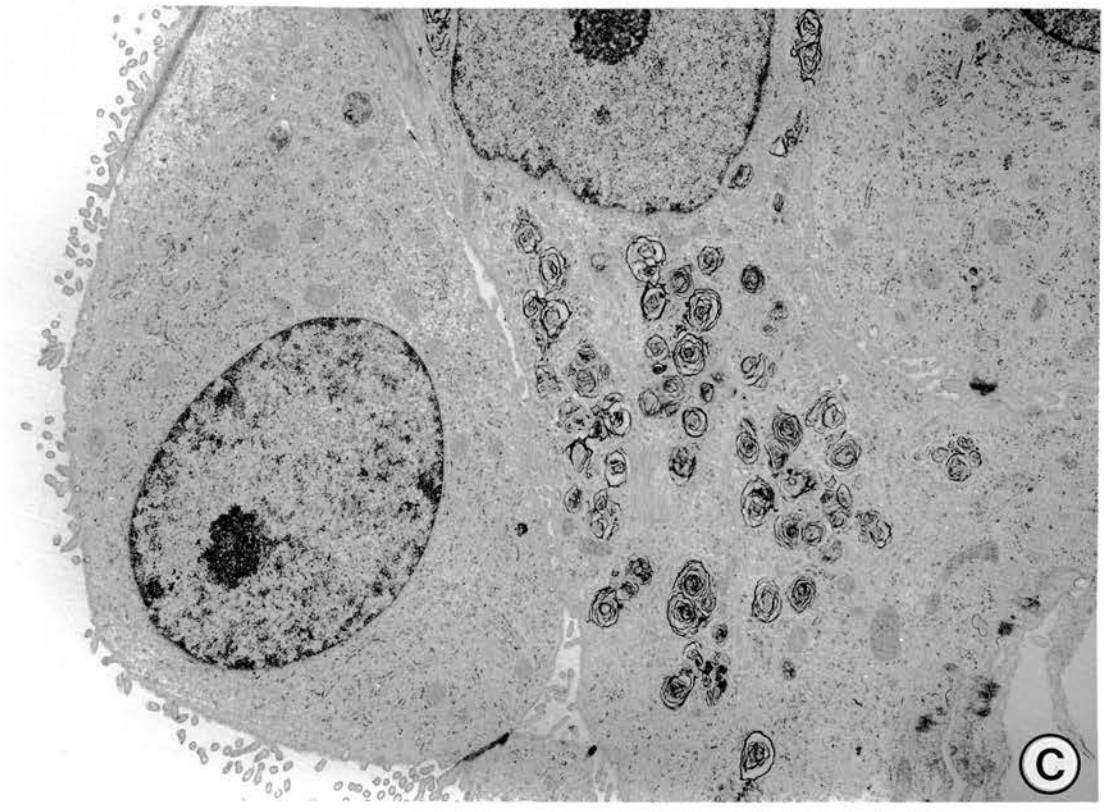
The bromhexine HCl-treated cells (Fig. 5.2) possessed microvilli and cells were joined by prominent desmosomes. The cytoplasm was rich in lamellar bodies which were seen singly or in groups. No glycogen granules were observed in the cytoplam of such cells. Nuclei were generally oval, with occasional indentation and the heterochromatin was uniformly dispersed throughout. The cells contained abundant ribosomes, both free and bound, and tonofilaments. Golgi apparatus and endoplasmic reticulum were well developed and mitochondria were

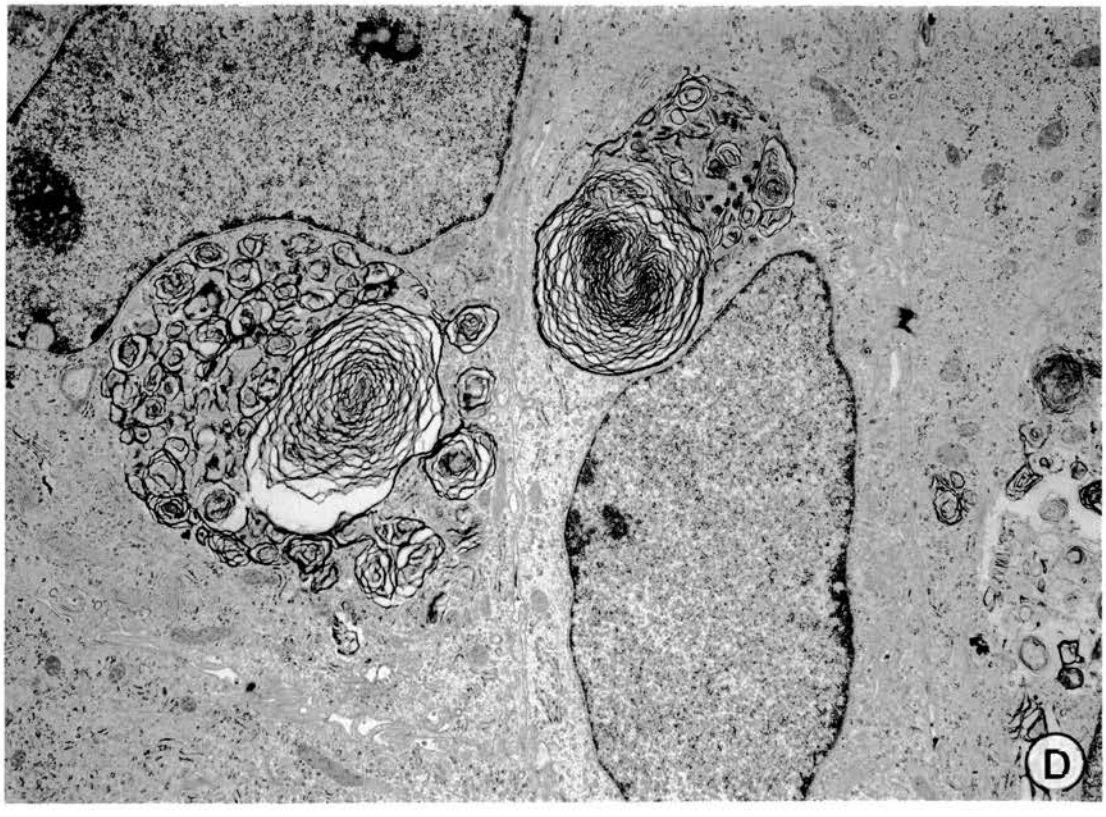
Fig. 5.2A,B,C,D

Electron micrographs of SPA cells after six days of *in vitro* treatment with bromhexine HCl. Numerous lamellar bodies are present in the cytoplasm of the cells. Glycogen deposits are absent. A), JS8 cells. Note the accumulation of lamellar bodies in the cells. B), higher magnification of the marked area (arrow) in A. Note the septate arrangement of the lamellar bodies. C and D, JS7 cells. Note the lamellar bodies in both and the formation of aggregates of lamellar bodies in D. A, x 4000; B, x 40000; C, 6000; D, 8000.


A


B

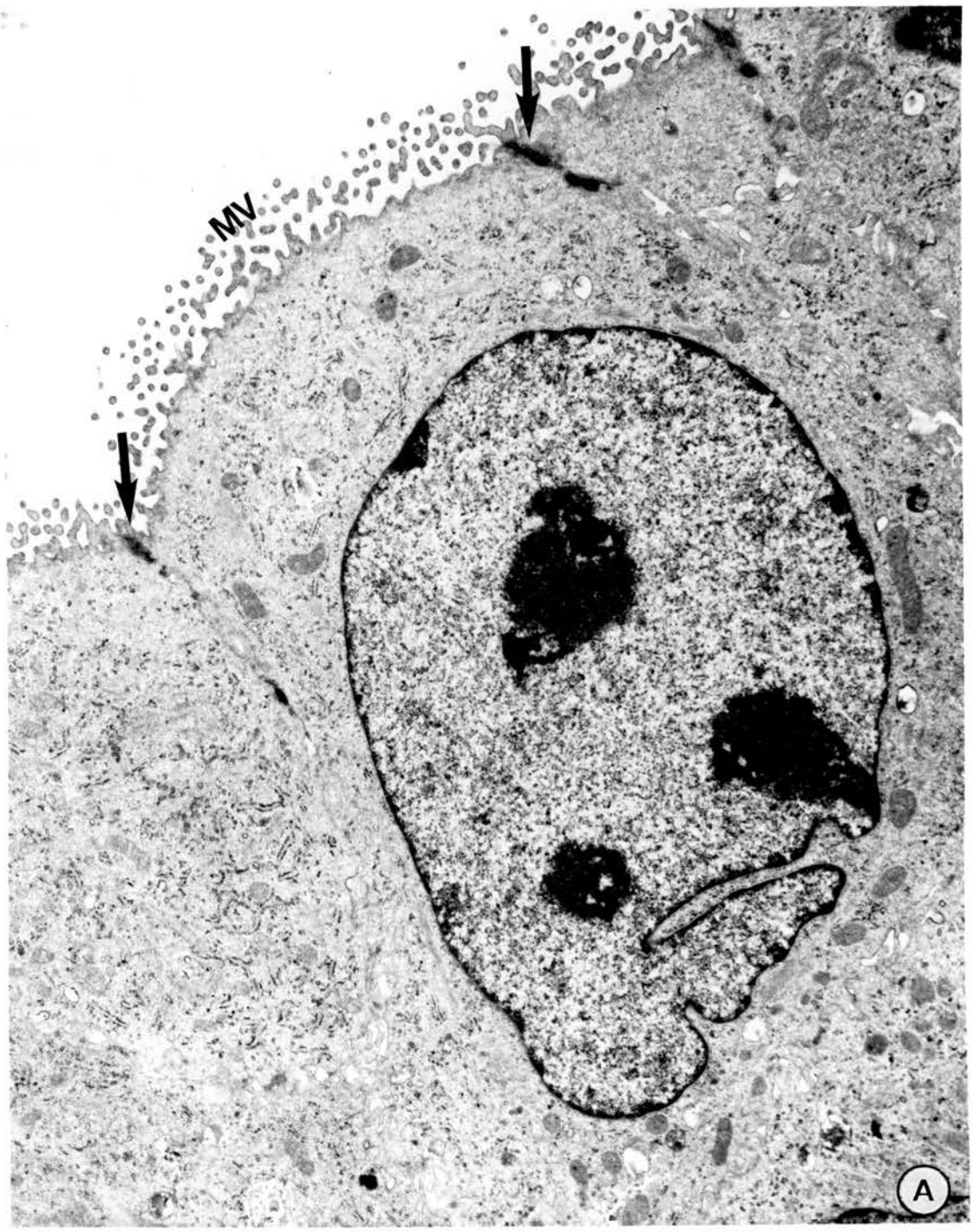

C

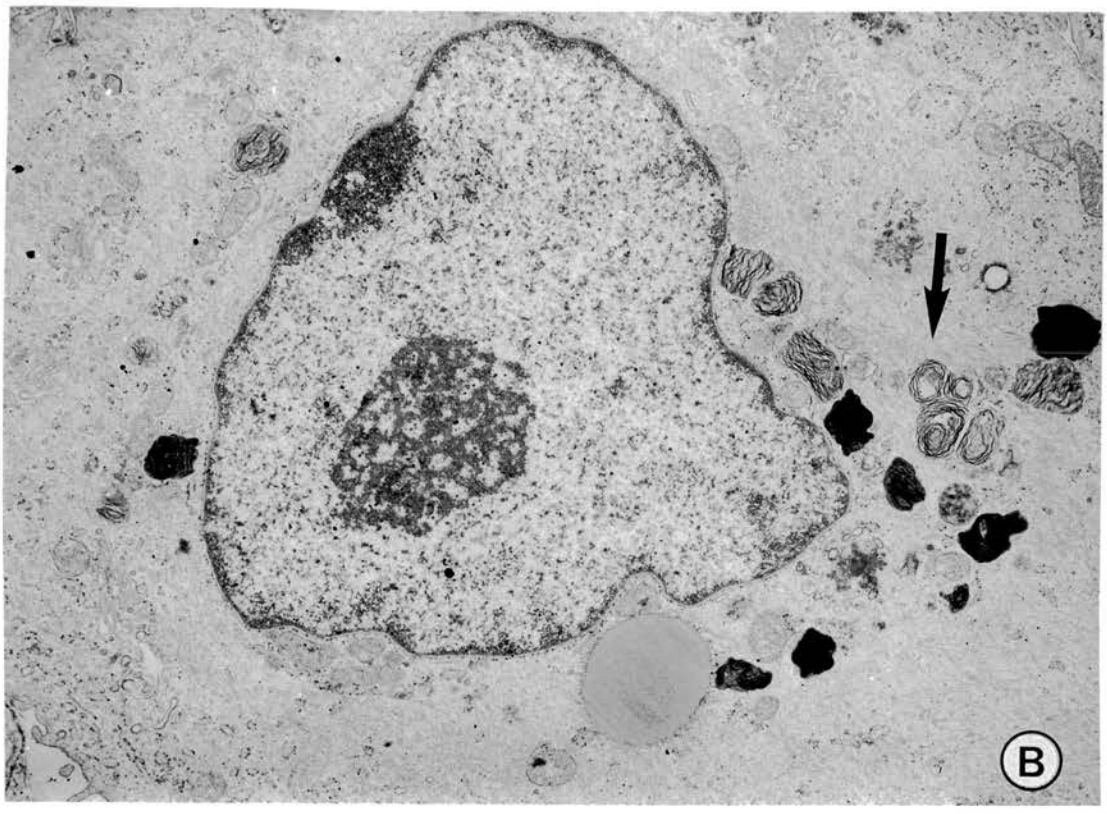

D

numerous. The untreated JS8 cells showed no evidence of lamellar bodies (Fig. 5.3A). Untreated JS7 cells exhibited lamellar bodies but these were smaller than bromhexine HCl treated cells (Fig. 5.3B,C). Prednisolone-treated cells (Fig.5.4) contained the usual cell organelles, such as microvilli, desmosomes, tonofilaments, endoplasmic reticulum and Golgi apparatus, but no lamellar bodies were demonstrated. Glycogen deposits were occasionally found in small amounts in some cells.

Fig. 5.3A,B,C

Electron micrographs of untreated SPA cells. A, JS8 cells. Note the cells showing the features of epithelial cells such as microvilli (MV) and desmosomes joining the cells (arrows). B, a cell of JS7 line containing lamellar bodies. C, a higher magnification of the area marked by arrow in B. A, x 9000; B, x 10000; C, 40000.

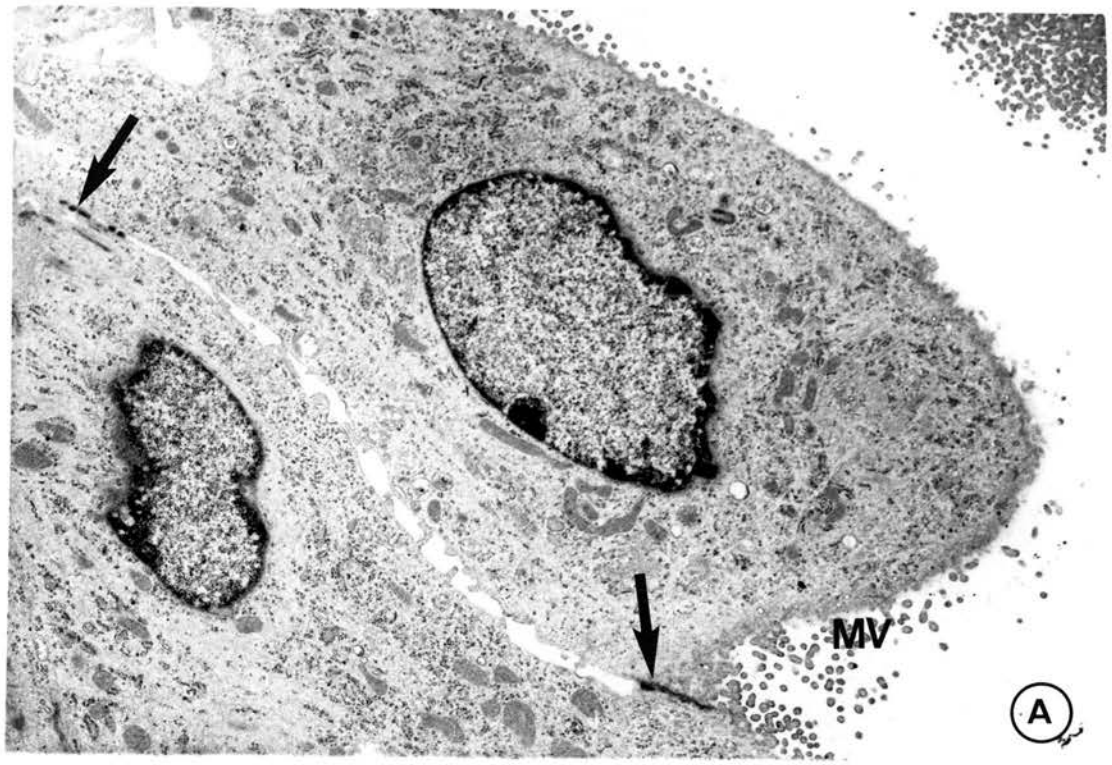

MV
A

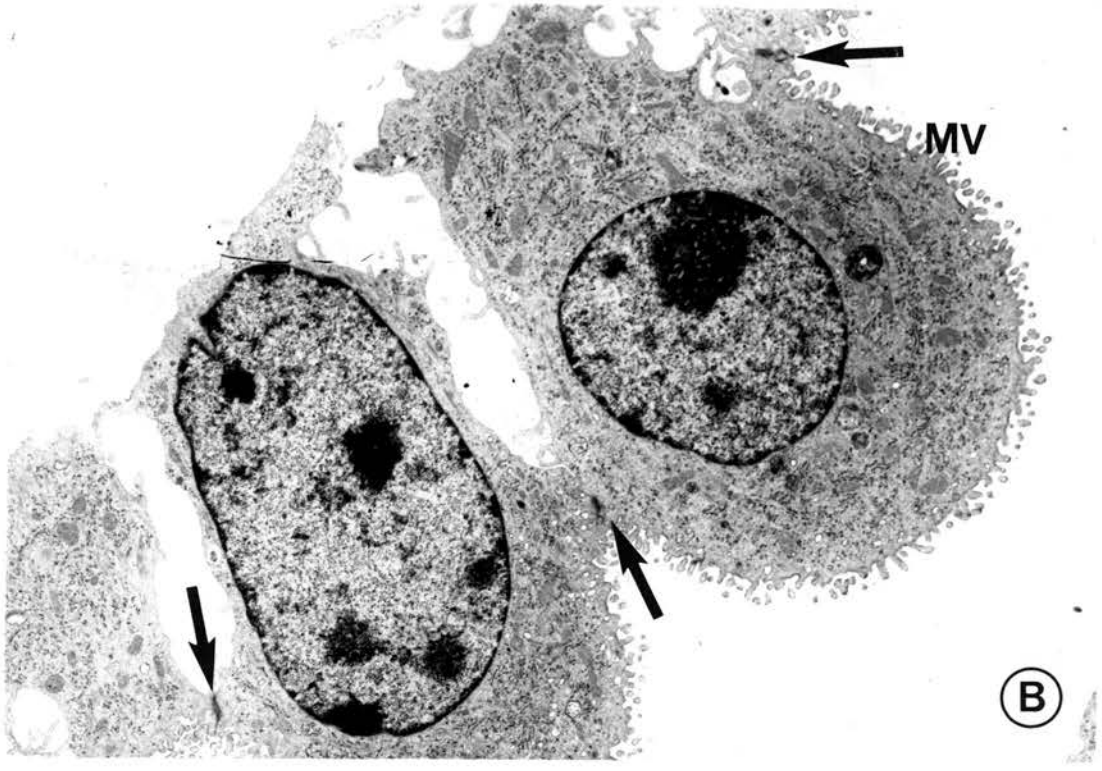

B


C

Fig. 5.4A,B

Prednisolone treated cells. A, JS8 and B, JS7. Note cells maintained their epithelial characteristic namely, microvilli (MV) and desmosomes (arrows). No lamellar bodies are seen. A, x 6000; B, 6000.


MV
A


MV
B

DISCUSSION

Prednisolone and bromhexine HCl both affected cultured SPA cells, but in a number of different ways.

With prednisolone treatment of JS7 and JS8 cell lines, there was a change in morphology and growth parameters *in vitro*. The saturation densities and plating efficiencies of treated cells were reduced when compared to untreated cells. In addition, cells changed from a pavement to a swirling arrangement and were devoid of lamellar bodies. In contrast, bromhexine HCl had little effect on gross cell morphology and not only increased the number and size of lamellar bodies in the treated cells but also reversed the lamellar body deficiency of JS8 cells. Findings regarding bromhexine HCl are in agreement with the observations of Bela and Angela (1984) who reported in an electron microscopic study that bromhexine HCl caused the appearance of lamellar bodies in human fetal lung *in vitro*. But the experiments with prednisolone are at variance with reports indicating that steroids can enhance the presence of lamellar bodies. In the present study, the presence of lamellar bodies has been taken as an indicator of the functional competence of the cells with regard to surfactant production. Therefore, in the absence of functional studies, such as choline chloride incorporation, the mechanisms underlying the effects of prednisolone and bromhexine remain speculative.

Biochemical and electron microscopic observations have shown that many agents such as pilocarpine, thyrotropin-releasing hormone, oestrogen, amiodarone hydrochloride and iprindole, modulate the function of type II alveolar cells. Some of these stimulate synthesis of surfactant, while others may influence its secretion (Goldenberg et al, 1969; Rooney et al, 1979; Khosla and Rooney, 1979; Khosla et al, 1981; Heath et al, 1985; Martin and Kachel, 1987). These authors indicated that the above agents caused an increase in the lamellar bodies and stimulated surfactant production. Therefore, the increase in number of lamellar bodies after bromhexine hydrochloride treatment could be due to enhanced synthesis. Another possibility, in addition to increased synthesis of surfactant in these cells, is that the secretory function of surfactant may have been disrupted as a result of transformation or during the course of cellular adaptation to an in vitro environment. However, this is unlikely since it was demonstrated in Chapter 4 that synthesis and secretion of disaturated phosphatidylcholine by JS7 and JS8 cells was low compared to that of freshly isolated type II alveolar cells (Mason et al, 1977b).

A further explanation is possible. Experimental studies have shown that treatment of rats and mice with amiodarone results in phospholipid accumulation in type II alveolar cells (Costa-Jussa et al, 1984). Heath et al (1985) also studied the effect of amiodarone

on adult rat lung using electron microscopy and biochemical assays. Their results showed that amiodarone caused a huge increase in size and number of lamellar bodies per cell and an increase in the total phospholipid content particularly phosphatidylcholine. The increase in phospholipid was associated with inhibition of lysosomal phospholipases A1 and A2. The authors suggested that amiodarone is a potent inhibitor of phospholipases and concluded that this block of phospholipid catabolism was the main cause of phospholipid accumulation. Similarly, phospholipid or DSPC accumulation in cultured L-2 alveolar cells in response to iprindole has also been reported (Martin and Kachel, 1987). It was suggested that iprindole may prevent phospholipid degradation by inhibiting phospholipase-A activity. Therefore, in view of the above observations, it is possible that, bromhexine HCl used in the present study, acted in a similar manner by preventing the degradation of phospholipids within SPA cells.

The present study indicates that prednisolone has an inhibitory effect on lamellar body formation. The mechanism whereby the lamellar bodies of prednisolone treated cells are lost is not known. However, recent studies indicate that cloned human fetal type II pneumocytes secrete phospholipase A2 (Lynn et al, 1979, cited by Batenburg, 1980), and such activation of phospholipase activity could account for the effect of prednisolone on JS7 and JS8 cells. However, it should be

pointed out that in other *in vivo* and *in vitro* studies, prednisolone appeared to have the opposite effect i.e it induced cell differentiation and increased lamellar bodies (Wang *et al*, 1971; Adamson and Bowdon, 1975; Adamson and Klass, 1976). One outstanding difference between the present experiments and previously published reports is that most previous studies were conducted *in vivo* or used organ cultures. In these latter systems the effects of administered steroids were complicated by secondary events in the animal or influences from other cell types. Evidence was presented that glucocorticoids may not act directly on the developing pulmonary epithelium, but rather upon the pulmonary mesenchyme, where they induce synthesis and secretion of fibroblast-pneumocyte factor which, in turn, stimulates DSPC synthesis by the alveolar type II cell (Smith, 1978). Thus, this is a further example of mesenchymal modulation of steroidal effects on epithelium (Lasnitzki and Mizuno, 1979). In the present study the cells used were free from fibroblasts, therefore, the absence of fibroblasts may have contributed to the failure of prednisolone to stimulate the SPA cells to differentiate in culture. However, this aspect remains rather unclear although the effect of prednisolone on choline incorporation has been studied previously in mouse lymphoma grown *in vitro* by Story *et al* (1973). They found that incorporation of choline into phosphatidylcholine was inhibited following exposure of

cells to prednisolone and that this inhibition was proportional to the concentration of steroid.

In the present studies it was observed that as lamellar bodies appeared, there was an associated decrease or disappearance of glycogen. This observation agrees with previous studies on fetal development which showed the same effect in type II pneumocytes (Adamson and Bowdon, 1975). Observations such as these have given rise to the opinion that glycogen may be involved in pulmonary phospholipid synthesis (Maniscalo et al, 1978). Such a hypothesis is supported by studies which have shown that agents such as corticosteroids, which stimulate lung surfactant production, also cause a decrease in lung glycogen content (Rooney et al, 1979) whereas agents such as insulin, which inhibit lung maturation and surfactant production, result in an increase in lung glycogen (Gross et al, 1978).

In the SPA cells, therefore, the disappearance of glycogen and the appearance of lamellar bodies in response to bromhexine HCl may be indicative of increased phospholipid synthesis.

In the present study, the changes in cellular morphology were consistent with those previously described for Chinese hamster ovary cells treated with testosterone or hydrocortisone treated cells of mouse lung adenomas (Hsie and Puck, 1971; Stoner et al, 1975). In both studies it was found that steroids caused conversion of the cells from an epithelial to a fibroblast-like

appearance. Hsie and Puck (1971) suggested that testosterone acts by promoting organization of microtubules from protein monomers, thereby resulting in cellular elongation. The same conclusion was reached on similar grounds by Stoner et al (1975) with respect to mouse lung adenoma cells treated with hydrocortisone. Because the alteration in phenotype of SPA cells treated with prednisolone resembles those described for Chinese hamster ovary cells and mouse lung adenomas, it would appear reasonable to believe that prednisolone acted in a similar way.

The morphological changes noted in culture in the presence of prednisolone may represent either altered morphology of the general cell population or selection of a particular subpopulation of cells. The hypothesis that these changes were due to cell adaptation to prednisolone rather than selection is supported by the observation that after removal of prednisolone, the cells reverted to their original morphology and growth characteristics.

The results in this chapter indicate that these SPA cell lines may provide a valuable model to study events in differentiation of type II pneumocytes and provide a means to elucidate the mechanisms regulating surfactant synthesis and secretion. Conceivably, this system ultimately may prove useful in screening various drugs for their potential value in assessing pharmaceutical agents.

CHAPTER 6

GROWTH AND BEHAVIOUR OF ESTABLISHED SPA CELL LINES IN ATHYMIC NUDE (nu/nu) MICE

INTRODUCTION

Flanagan (1966) first described a strain of mouse which, when homozygous for the nude (nu) gene, is both hairless and congenitally athymic (Pantelouris, 1968). These nu/nu mice were used successfully by Rygaard and Povlsen (1969) for transplantation of human adenocarcinomas of the colon and rectum. The histopathology of the transplanted tumours resembled that of the original. Furthermore, it was demonstrated that during passage from mouse to mouse the histological appearance of the transplanted tumour remained constant (Povlsen and Rygaard, 1971 & 1972). Since these earliest reports, nude mice have been utilized for the growth of a wide range of human tumours (Price et al, 1975; Schmidt and Good, 1975; Shimosato et al, 1976; Mickey et al, 1977; Shimosato et al, 1979; Giovanella et al, 1978 & 1979; Tamai et al, 1980; Wennerberg, 1984), some of which can even induce invasive and metastasizing neoplasms in the recipient mice (Giovanella et al, 1972; 1973; 1974; Hata et al, 1978; Takahashi et al, 1978; Giovanella et al, 1978; Sharkey and Fogh, 1979; Mattern et al,1980).

The use of nude mice has not been limited to the study of human tumours, but adapted with equal success for studies of animal neoplasms. Oughton and Owen (1974) successfully transplanted several types of canine tumours, such as osteosarcoma cell culture, melanoma cell lines and cell cultures or cells directly obtained from spontaneous canine tumours (osteosarcoma, mammary adenocarcinoma, lymphosarcoma, transmissible venereal tumour). Other authors have described transplantation of canine, feline, equine and bovine tumours, and in each case, reported that the transplanted tumour retained the histological features of the original (Inoue et al, 1980; Thomas et al, 1983; Norval et al, 1984; Ladiges and Hoosier, 1980; Norval and Maingay, 1985; Pardini et al, 1986; Al-Yaman and Willenborg, 1984).

Despite the success of these many reports, some human and animal tumours have failed to grow in nude mice unless the mice were subjected first to whole-body irradiation. For example, transplantation of a human retinoblastoma was unsuccessful unless the mice were exposed to whole-body irradiation with 500 rads, three days before inoculation (Shimosato et al, 1979). Similarly, one osteosarcoma and five bladder adenocarcinoma cell lines which had been shown previously to be non-tumour producing, grew subcutaneously as malignant tumours in whole-body x-irradiated mice (Giovanella and Fogh, 1985). In the veterinary field, a canine lymphosarcoma grew successfully in whole-body

X-irradiated nude mice, and was subsequently transplanted to both irradiated and non-irradiated nude mice (Morgan et al, 1978). Similarly both bovine lymphosarcoma and bovine lymphoid cells infected with Theileria parva, produced tumours in all x-irradiated athymic mice, whereas, no tumours developed in any of the non-irradiated mice (Irvin et al, 1975a; 1975b; 1977). From the above example, it is clear that nude mice are now well established as a means of evaluating, in vivo, the tumourigenic potential of cells that have been derived from human or animal tumours and grown in vitro as primary cultures or cell lines (Price et al, 1975; Stiles et al, 1976). An additional application of this technique in the field of oncology has been to use the mouse as a vehicle for mass production of differentiated cells, with the transplanted tumours maintaining their original functional activities (Freedman and Shin, 1974; Ozzello et al, 1974; Pesce et al, 1977; Fisher and Paulson, 1978; Rao et al, 1980).

Nude mice have not been used extensively to study the biology of SPA. However, in two recent reports, tumour cells or minced tissue from sheep with pulmonary adenomatosis (SPA) were shown to produce cystic tumours in nude mice (Verwoerd et al, 1977; Zimber et al, 1984). Microscopic examination revealed the cysts were lined by a single layer of epithelial cells and that the tumours showed a close correlation in their histological appearance with the original tumour in sheep.

The present studies were undertaken for a number of reasons. First, in the preceding chapters many of the features associated with transformed cells, which include alteration in cell morphology, change in growth properties, loss of contact-inhibition, increased plating efficiency, increased saturation density, abnormal karyotype, and anchorage-independence, were studied only in vitro. The capacity of cells to grow in nude mice has emerged as the most reliable and consistent criterion for assessing the transformed characteristics of cells (Freedman and Shin, 1974) and such information would complement the results gained from the in vitro studies. Furthermore, the information available from previous studies of SPA cells (Verwoerd et al, 1977; Zimber et al, 1984) did not show that cells forming the tumour in nude mice retain morphologic and ultrastructural features of the original SPA cells, nor even that they are sheep cells and not transformed mouse cells. In this study the four cell lines described in Chapter Four were inoculated into nude mice to examine aspects not covered by previously published work. Emphasis was placed on morphology, histopathology, latency period, and ultrastructural features.

MATERIALS AND METHODS

Mice:

For heterotransplantation, 4-6 week old male and female athymic nude mice (C.B.A. background) were used. They were obtained from MRC, Clinical Research Centre, Northwick Park Hospital, Watford Road, Harrow, Middlesex HA1 3VJ.

Mice were maintained throughout the experiments in a specific pathogen-free environment in flexible vinyl film isolators (Fig. 6.1). They were held singly or in groups according to the design of the experiments and were used for experiments after an acclimatisation period of about seven days.

Medium:

All cell lines were grown in Ham's F12K medium supplemented with 10 percent fetal bovine serum.

Cell Lines:

A). SPA cell lines

Each of the four cell lines derived from SPA tumours, JS7, JS8, JS14 and JS15 described in Chapter 4, were used.

B). Control cell lines:

The A549 tumour-cell line was initiated from a human adenocarcinoma and is purported to originate from type II pneumocytes (Lieber et al, 1976).

Fig. 6.1

Positive-pressure flexible vinyl film isolator used to maintain nude mice in a specific pathogen-free environment. The isolator was placed in a controlled environment room.

The NBL12 cell line, was initiated from the lung of an adult female bat (Kniazeff et al, 1965). The cells were predominately epithelial-like. In our laboratory this cell line was found to grow in soft agar.

A549 and NBL12 were chosen as control cell lines because both were originated from lung and demonstrated properties of transformed cells. Their growth rate and tumour type were compared with those of the SPA cell lines.

Transplantation to nude mice

For injection into nude mice, cells were harvested by trypsinization, centrifuged and washed in growth medium. The number of viable cells was determined as described in General Materials and Methods. Mice were injected subcutaneously in the interscapular or thoracic region with 0.2 ml of cell suspension containing 10^7 viable cells. They were examined daily for tumour growth and sacrificed by neck dislocation after 3-6 weeks. Tumours were dissected free from the skin and subcutis and portions processed for histopathology and electron microscopy.

Some tumours were removed and immediately processed for the re-establishment of cells in vitro (see Chapter 7). Cells lines were scored as tumourigenic if palpable nodules that appeared at the site of injection were confirmed by histopathological examination as showing an appearance consistent with SPA tumour.

Effect of cell number on tumour incidence in mice

Several reports indicate that the duration of the latency period and tumour induction in nude mice, depends on the number of cells inoculated with that particular strain (Franks et al, 1976; Freedman et al, 1976). Therefore, to determine the minimum cell dose required for tumour induction and its effect on the lag period, JS7 was taken as a representative test line for SPA cell lines. Cells were inoculated into nude mice at doses of 10^7, 10^6, 10^5, 10^4 cells. Each cell inoculum was injected into three mice (two males and one female). Tumour development and measurement were recorded as described below.

Kinetics of tumour growth in vivo

For the determination of the in vivo growth rate of SPA and control cell lines, a group of five nude mice of mixed sexes were used for each individual cell line. Each mouse was injected subcutaneously with 10^7 cells of each line, and then was examined daily. Visible tumours were measured with calipers in two dimensions at three day intervals and the tumour volume was calculated according to the formula: Length x (Width)^2 x 0.4 (Attia and Weiss, 1966). The mean tumour volume for each group was recorded and plotted against time.

Histology:

Histological stains:

A). Haematoxylin and eosin

Tissues from tumours were fixed in Baker's calcium-formol and processed as described in General Materials and Methods.

B). Periodic acid-Schiff (PAS)

For the detection of glycogen in tumours induced by the SPA cell lines, sections were prepared and stained by the periodic acid-Schiff (PAS) reaction as described by McManus (1946) and modified by Pearse (1959) (cited by Drury and Wallington, 1967). Diastase digestion of sections prior to PAS staining was undertaken as further confirmation of the presence of glycogen.

Diastase treatment was achieved by incubating sections with 0.1% malt diastase in distilled water for 30 minutes at 37°C.

C).Alcian-blue

Tissues from nude mice tumour were fixed immediately in Baker's calcium-formol, embedded in paraffin, sectioned, and stained by Alcian blue at pH 2.5 (Spicer and Lillie, 1959)

Scanning electron microscopy

Blocks of tumour were cut, sectioned and then processed as described in General Materials and Methods.

Transmission electron microscopy

For transmission electron microscopic studies, fragments of nude mice tumours were taken immediately after excision of the tumour from mice and processed as described in General Materials and Methods.

Negative staining

This procedure was employed to detect extracellular virions. A pool of cystic fluid was clarified by centrifugation at 10000 xg for 30 minutes at 4°C. Formvar/carbon coated grids were placed on a drop of 0.1% poly-L-lysine for 30 seconds, drained and transferred onto a drop of cystic fluid for 5 minutes. The grid was washed several times with distilled water and stained with 1% potassium phosphotungistic acid pH 7.0 and examined in a Siemens Elmiskop 1A electron microscope.

RESULTS

Transplantation to nude mice

A total of 37 nude mice were transplanted with four different SPA cell lines as indicated in the experimental design. The results of these experiments are presented in Table 6.1.

All attempted transplantations of JS7, JS8 and JS15 cell lines were successful and produced loosely bound cystic tumours at the site of injection (Fig.6.2). It is evident from Table 6.1 that these three cell lines induced tumour growth in 100% of the mice. The latency period between transplantation and first positive evidence of tumour growth was 3 to 5 days and the take rate was similar in both male and female recipient mice. The mice remained clinically healthy despite the growth of the tumour. No signs of spontaneous regression or surface ulceration of the tumours were observed even when mice bearing tumours were kept up to three months after inoculation.

In all cases the gross appearance and nature of the tumours formed by the three cell lines at different passage levels remained similar. The tumours were cystic, which was evident from external palpation and the viscous, straw-coloured fluid was obtained by aspiration. In general 0.5-1.5 ml of fluid was obtained from each single tumour. This fluid remained for the duration of the experiments in all mice. Fluid from cystic tumours was

Table 6.1

Tumour induction in nude mice

Cell line	Passages tested	Type II pneumocytes (%)	No.tumours/ no.animals inoculated	Latency (days)	Efficiency (%)
JS7	6	90	3/3	5	100
JS7	46	85	5/5	5	100
JS7	116	ND	3/3	4	100
JS8	8	8	2/2	5	100
JS8	52	ND	5/5	5	100
JS8	103	ND	3/3	4	100
JS14	9	15	1/6	5	17 *
JS14	10	ND	5/5	4-5	100
JS15	15	87	5/5	3	100

ND: not done

* All tumours regressed.

Fig. 6.2

Nude mice with subcutaneous cystic tumours, resulting from the inoculation of 10^7 SPA tissue culture cells (JS15, passage,15). Tumours grew to this size within 4 weeks.

collected and light microscopy showed that it contained single cells or groups of free cells. Trypan blue exclusion revealed that a high proportion of cells were dead, although some viable cells were present and cultures were initiated from these (Chapter 7).

The excised tumours were soft, well-defined, loosely bound to the surrounding tissues, and peeled easily from the skin and subcutis. There was no apparent blood vessel invasion from surrounding tissues.

In contrast, the JS14 cell line was least tumourigenic. All mice inoculated with this cell line developed nodules by 5 days and these increased in size for a further 6 days. However, in 10 of the mice, the nodules regressed spontaneously and in only 1 mouse did the nodule continue to grow. Histological examination of regressing nodules showed no evidence of SPA tumour cells. The regressing nodules were cyst-like containing autolysed cell debri (Fig. 6.3). The one successful heterotransplantation showed features typical of the other SPA cell lines in nu/nu mice.

Effect of cell number on tumour incidence in mice

The data presented in Table 6.2, indicate that tumour formation depends on the number of cells inoculated. An inoculum of 10^7 cells produced tumour growth in all of the injected mice within four days, whereas a dose of 10^6 cells induced a tumour in only 1/3 mice within 6 days. Cells failed to generate tumour growth at all if less than 10^6 cells were inoculated into each mouse.

Fig. 6.3

Section through a regressing nodule induced by the JS14 cell line. Note the fibrous capsule (arrow) surrounding autolysed cell debris. H & E stain, x 102

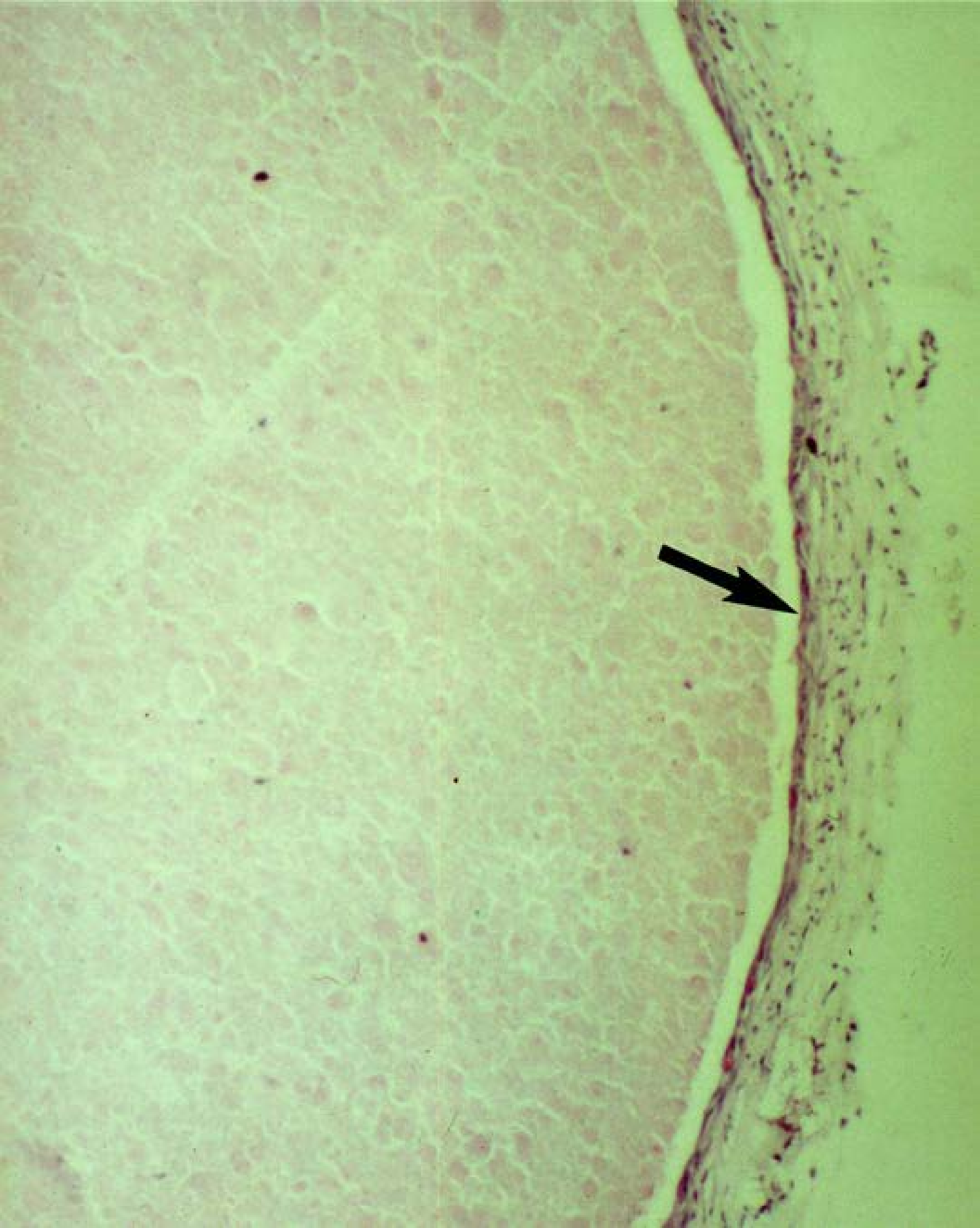


Table 6.2

Effect of JS7 cell dose on tumour induction in mice

No. of injected cells	Mice with tumours/ mice injected	Efficiency (%)
10^7	3/3	100
10^6	1/3	33.3
10^5	0/3	0
10^4	0/3	0

Kinetics of tumour growth in vivo

After injection of 10^7 JS7, JS8, JS14, and JS15 cells there was a period of latency during which time tumours were neither visible nor palpable. The latent periods for all cell lines were not different in males or females and for JS7 and JS8 did not change when high passage cells were used. The growth curve after a latency period of 3-5 days was characterized by an initial phase of rapid increase in tumour volume, followed by a phase of very slow growth, remaining in this phase until the tumours were removed (Fig. 6.4). Tumours derived from JS7, JS8, and JS15 cell lines had mean maximum sizes 43 days after transplantation of 856, 350, and 1476 cu.mm., respectively (Table 6.3).

The size of the tumour was accounted for mostly by the fluid content. Cells which secreted more fluid showed the greatest tumour size. Fluid secretion not only varied between cell lines, but also among nude mice inoculated with the same cell line as evidenced by large standard errors. Despite this individual variation, it was clear that JS15 cell lines induced tumours with more fluid than did the other three cell lines.

Following withdrawal of the fluid, there was total collapse of the cyst. Although fluid accumulated again and filled the cyst in 1-2 weeks, in no case did the cyst regained its original size. If aspirated for a second time even less fluid accumulated, and the resultant tumour was smaller than before.

Fig. 6.4A,B

A), Growth of SPA tumour cells in nude mice. Mice were injected with 10^7 cells on day 0 and the tumour volume measured at the indicated times. Each point represents the average tumour volume of 5 mice. B), growth curve of A549 and NBL12 cell lines conducted simultaneously. Standard errors have been omitted for clarity. (see appendix 2).

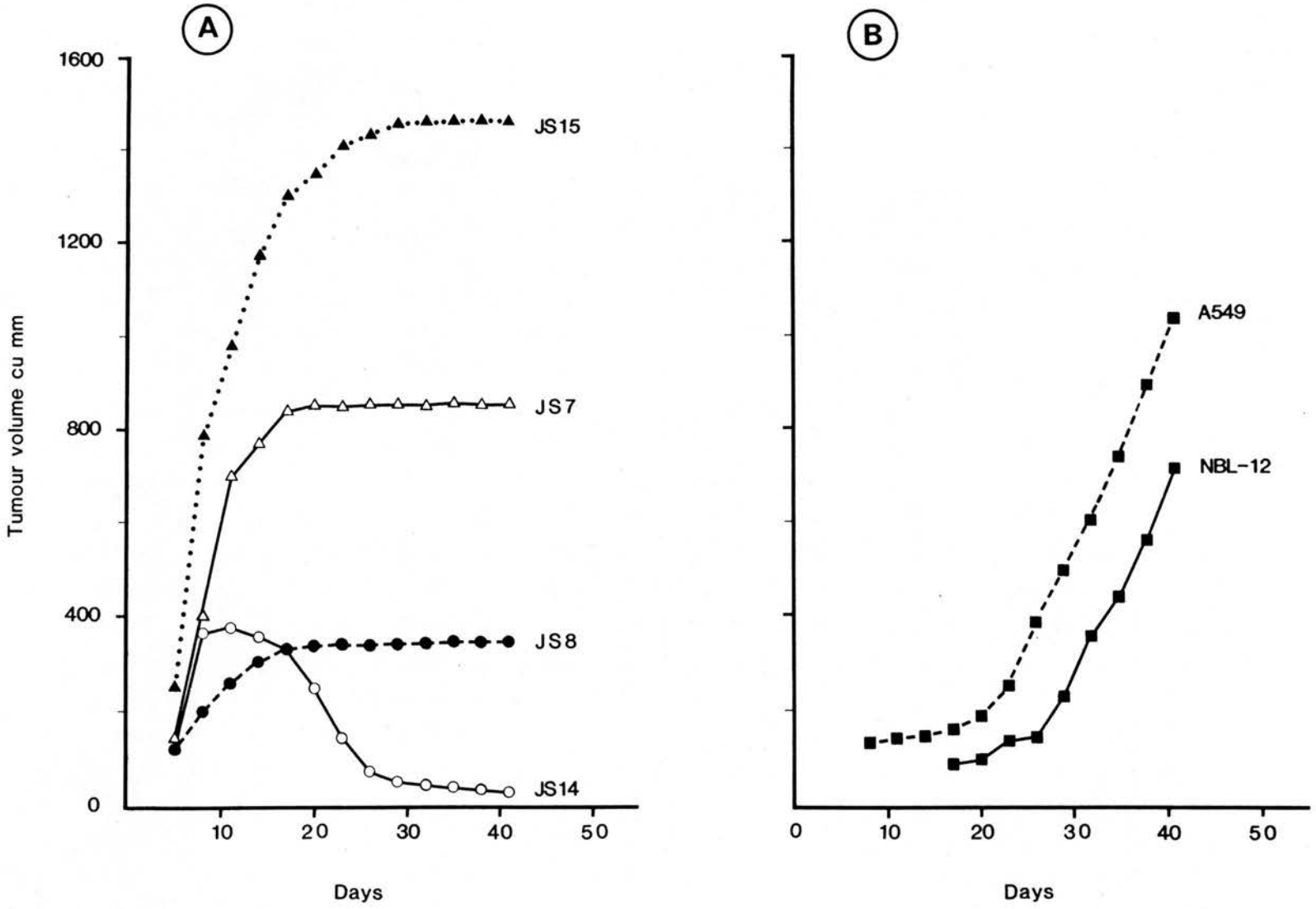

A
B
1600
1200
800
400
0
Tumour volume cu mm
JS15
JS7
JS8
JS14
10
20
30
40
50
Days
A549
NBL-12
0
10
20
30
40
50
Days

Table 6.3

Latency periods and maximum tumour volumes

Cell line	Passage tested	Latency (days)	Maximum volumes (cu mm)
JS7	46	5	856±267
JS8	52	5	350±60
JS14	9	4-5	#
JS15	15	3	1476±298
A549	38	7	1030±273
NBL12	82	14-16	709±282

* Figures represent the size of tumours 43 days after transplantation.

Tumour regressed

For the two control cell lines, tumour incidence was 100%, and the latent periods were 7 days for A549 and 14-16 days for NBL12. Thereafter, A549 and NBL12 cell lines demonstrated a growth pattern completely different from that of the SPA cell lines. The tumours that appeared after the period of latency, passed through a stage of slow growth for 26 and 20 days respectively. They then entered a phase of exponential and rapid growth, which was sustained until the tumour was excised.

Histology ★

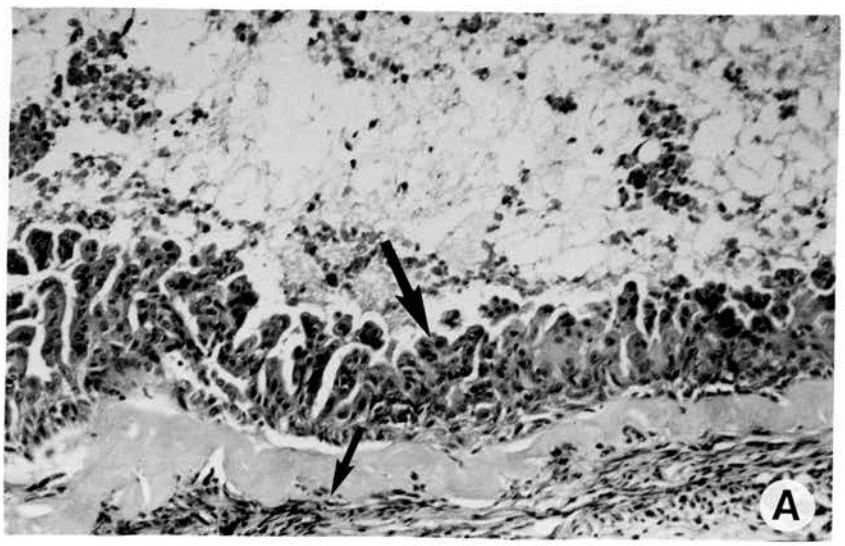
Characteristically, the tumours consisted of fluid-filled cysts enclosed by fibrous capsules. The cysts were lined internally by a simple or occasionally stratified epithelium composed of cuboidal cells with basally-situated vesicular ovoid nuclei, containing prominent nucleoli. Ingrowth of frond-like (Fig. 6.5) or polypoid (Fig. 6.6) structures arose at intervals from the cyst lining and projected into the central space. The surface epithelial layer of these structures was continuous with the cyst lining, but the cells covering the polyps tended to be more pleomorphic than those in the interpolyp area, and rounded or even polygonal forms were common. No differences in the morphology of the polyps were detected between tumours initiated by the different cell lines or different passes of the same cell line. In some polyps, acinar structures (Fig. 6.7) were seen, but it was not clear whether they were true acini, or sections through tubular invaginations from the polyp surface. In older tumours (6 weeks), there was a tendency for the cells covering the polyps to undergow necrosis and slough into the central space.

Varying amounts of PAS-positive granular material could be demonstrated in cells lining the cysts and cells comprising the tumour ingrowths (Fig. 6.8A). The amounts were often small and confined to the apical or lateral cytoplasm. The reaction was abolished by diastase pre-treatment (Fig. 6.8B), indicating that the

★Contributed by Mr. K.W. Angus

Fig. 6.5A,B

A), Section of tumour showing frond-like structures (large arrow) projecting into the central space of the cyst. Capsule of fibrous tissue (small arrow) enclosing the cyst. H & E stain; x 150; (B), same as in A, but at higher magnification. Note the epithelial morphology of cells forming the papillary structures. x 370.


A

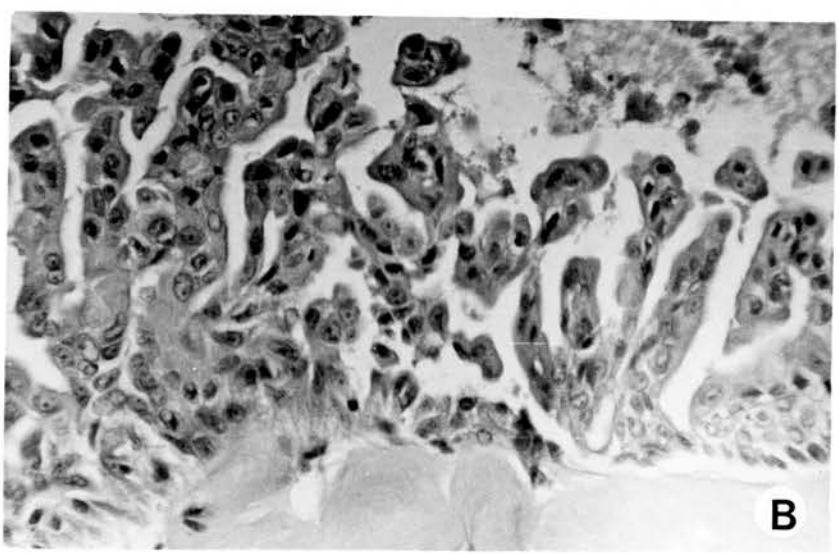
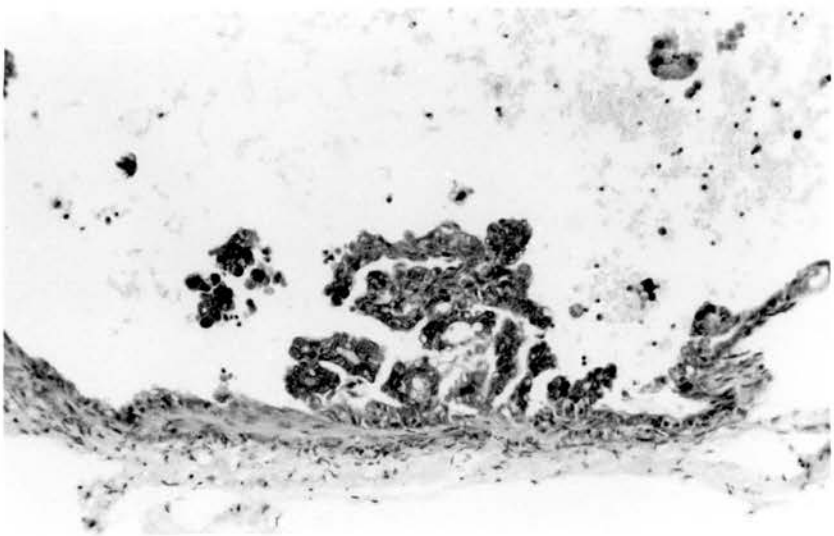

B

Fig. 6.6

Section of cystic tumour with polyp arising from the cyst lining and projecting into the central space. H & E stain, x 109

Fig. 6.7

Histological section of tumour with acinus-like structures. H & E stain, x 370



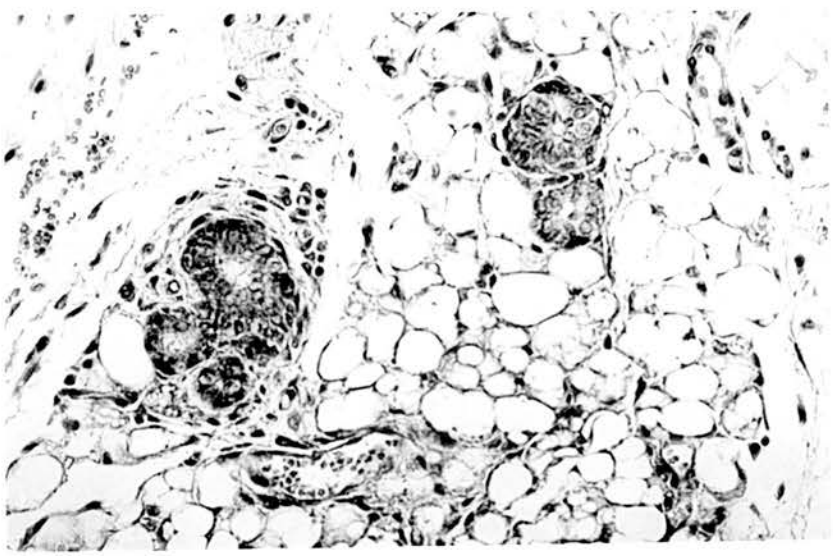
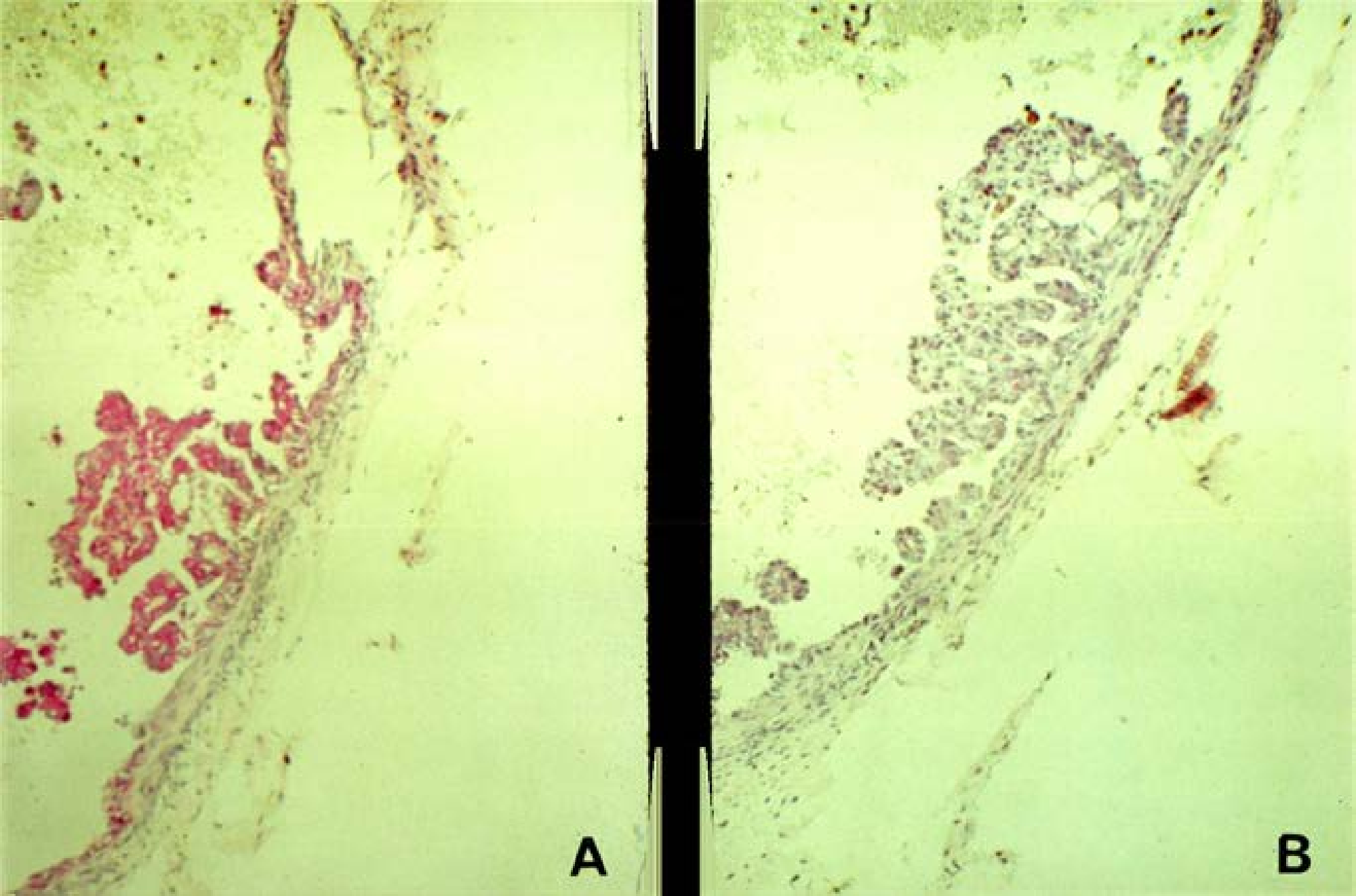


Fig. 6.8A,B

Histological section of tumour induced by JS7 cells in nude mice.

A). Stained by the periodic acid-Schiff method showing cells rich in PAS-positive material. x 90.

B). The Schiff reaction abolished by amylase pre-treatment, suggesting that the cells contained glycogen. x 90.


A
B

material probably was glycogen. In some tumours, epithelial cells containing Alcian blue-positive granules in addition to PAS-positive material were seen. These cells tended to be in short, discrete rows or islands amongst the other cells that did not stain with PAS or Alcian-blue. In JS15 only, there was evidence of some continuing cell multiplication, as indicated by the presence of mitotic figures.

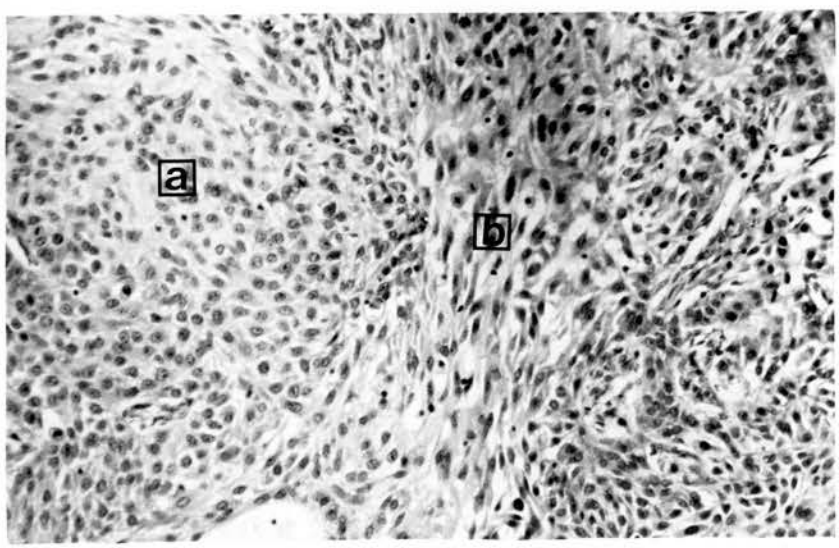
Control cell line A549 caused semi-solid tumours composed of two distinct cell populations (Fig. 6.9). Cells of epithelial derivation, possessing spherical nuclei and abundant cytoplasm rich in PAS-positive granules, formed large masses apparently enclosed by wide cords or palisades of intensely-stained fusiform cells resembling sarcomatous tumour cells. Within the tumour were large spaces containing a protein-rich material, erythrocytes and necrotic tumour cells. Control cell line NBL12 caused solid sarcoma-like tumours apparently composed only of darkly-stained spindle-shaped cells with ovoid nuclei, many of which were in mitosis (Fig. 6.10).

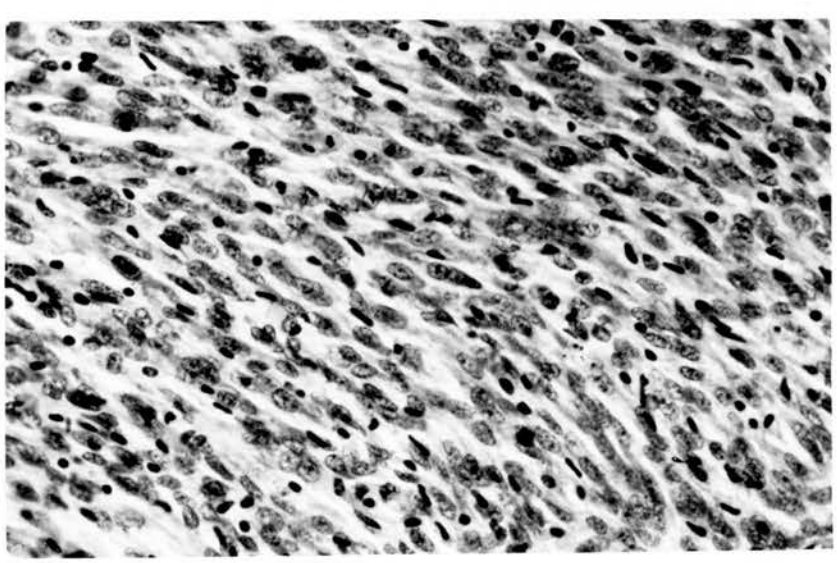
Fig. 6.9

Tumour section from transplant of A549 showing two different areas from same tumour. In a, cells are epithelial. In b, cells are fusiform, resembling fibroblasts. H & E stain, x 150

Fig. 6.10

Histology of NBL12 cells passaged in nude mice. The morphology is that of a sarcoma. Note the fusiform appearance of tumour cells.
H & E stain, x 150


a
b



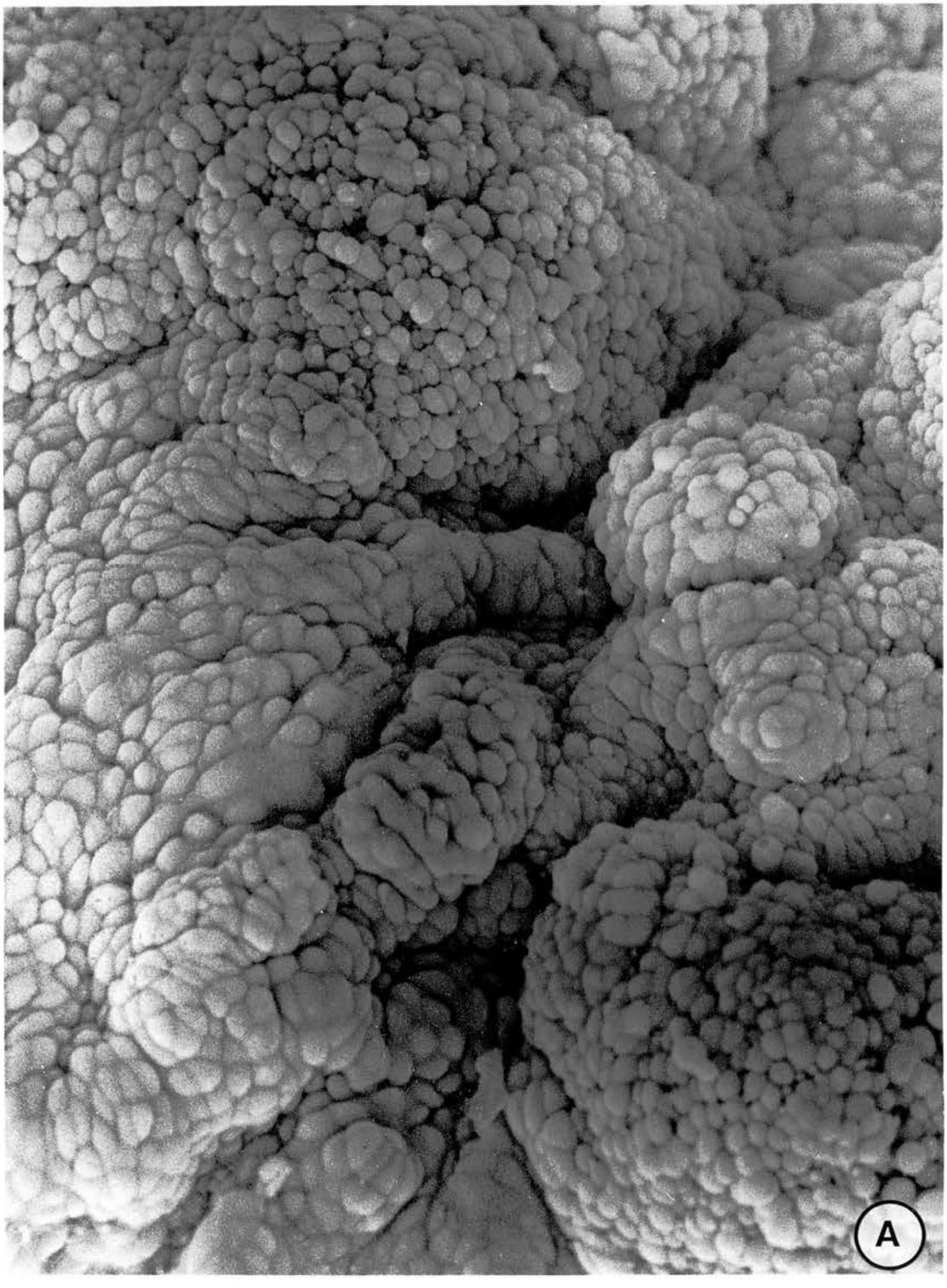
Scanning electron microscopy

When the tumours induced by cells of JS7, JS8, and JS15 were examined, the inner lining of the cyst demonstrated a convoluted base of cells with clearly defined boundaries (Fig. 6.11). The luminal surface of the cells comprising the base layer was entirely covered with numerous microvilli. The microvilli were short, blunt and of uniform size and distributed over the surface of the cells (Fig. 6.12).

At various points, buds or polyps were seen projecting into the lumen of the cyst (Fig. 6.13). Cells at the free tip of the polyps appeared to have microvilli with diverse morphologies (Fig. 6.14). Most of these microvilli were irregular, blunt or club-shaped and varied in size and shape and were more randomly distributed. Others were pointed and appeared longer. Some cells showed areas free from microvilli.

Fig. 6.11A,B

Scanning electron micrograph showing a portion of JS8 tumour growing in nude mouse. (A), cells comprising the base layer showing convolutions, (x 400). Note the well-demarcated cells (B), exhibiting numerous microvilli, (x 4000).


A

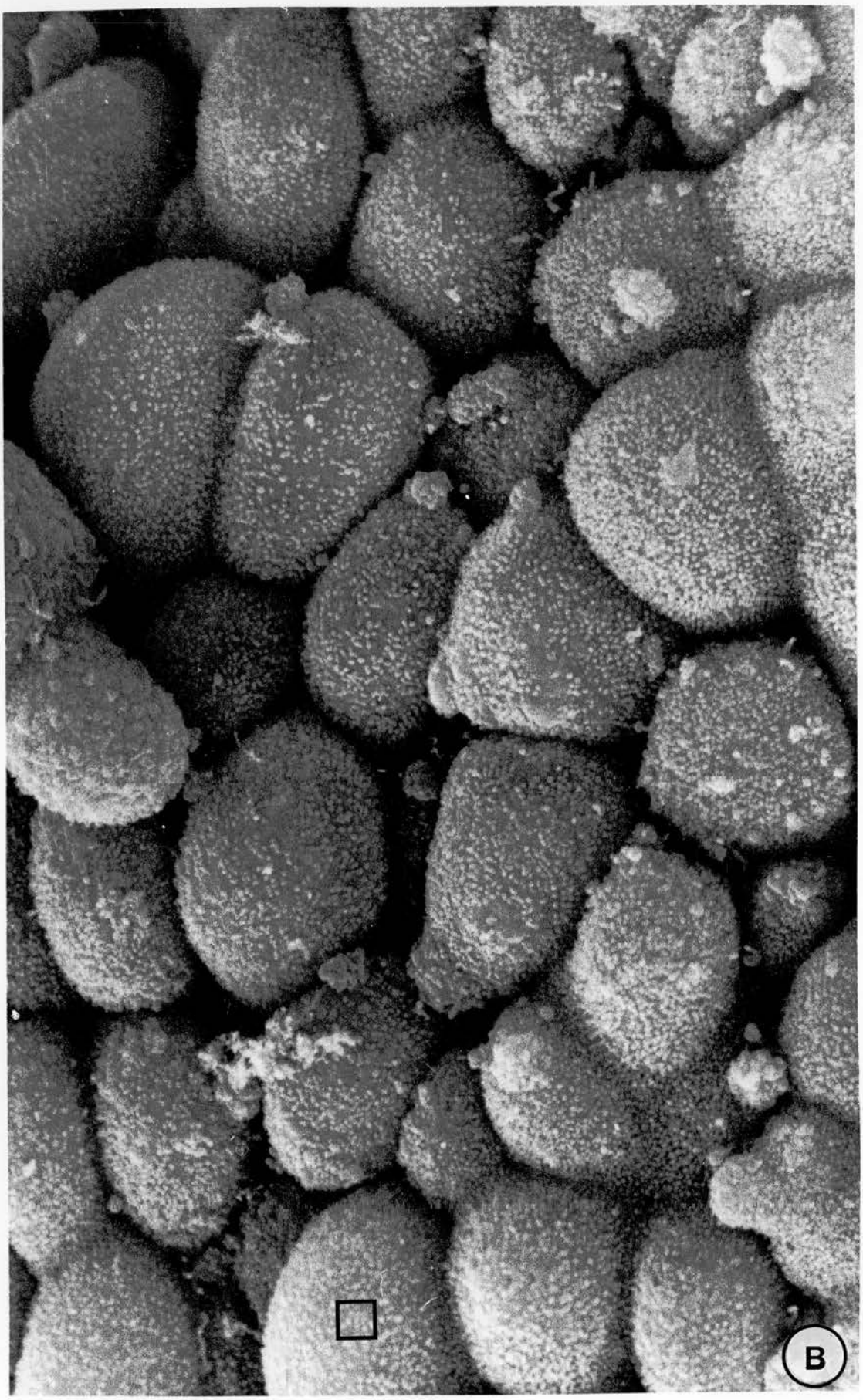

B

Fig. 6.12

Higher magnification (x 10500) of the cell marked by square in Fig. 5.11B. Microvilli are short and blunt-shaped.

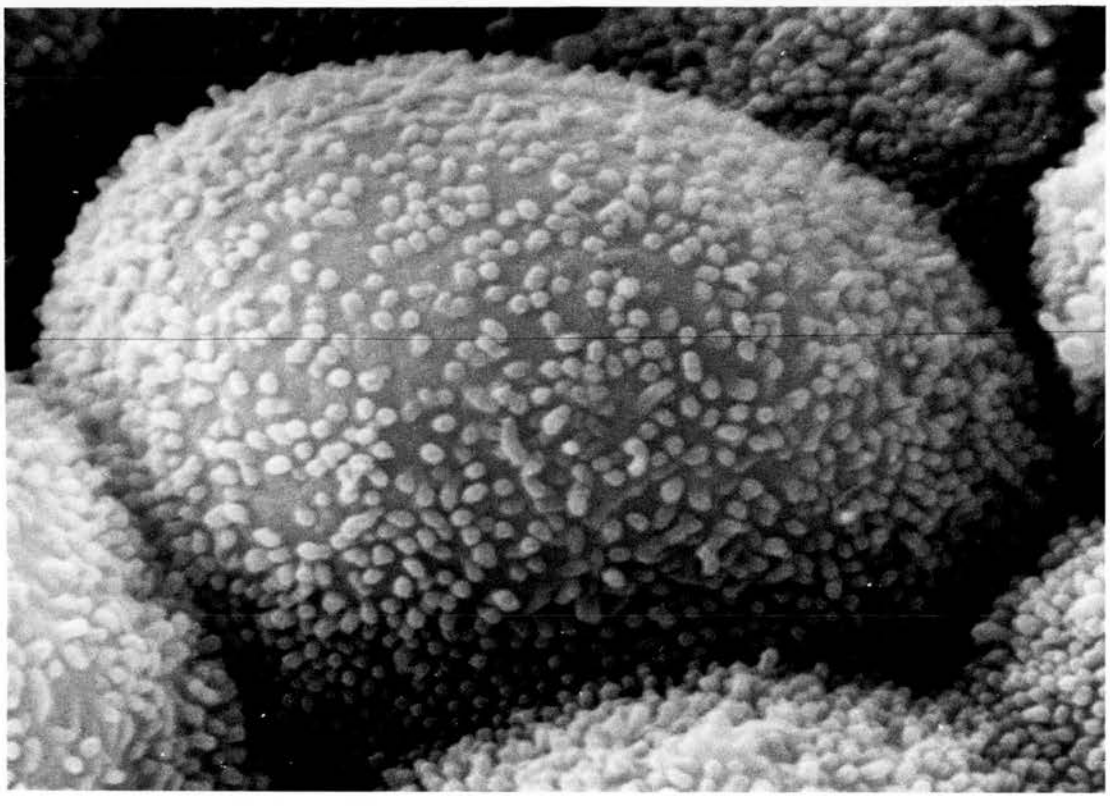


Fig. 6.13

Two polyps arising from the inner lining of tumour induced by JS8 cells (x 300).

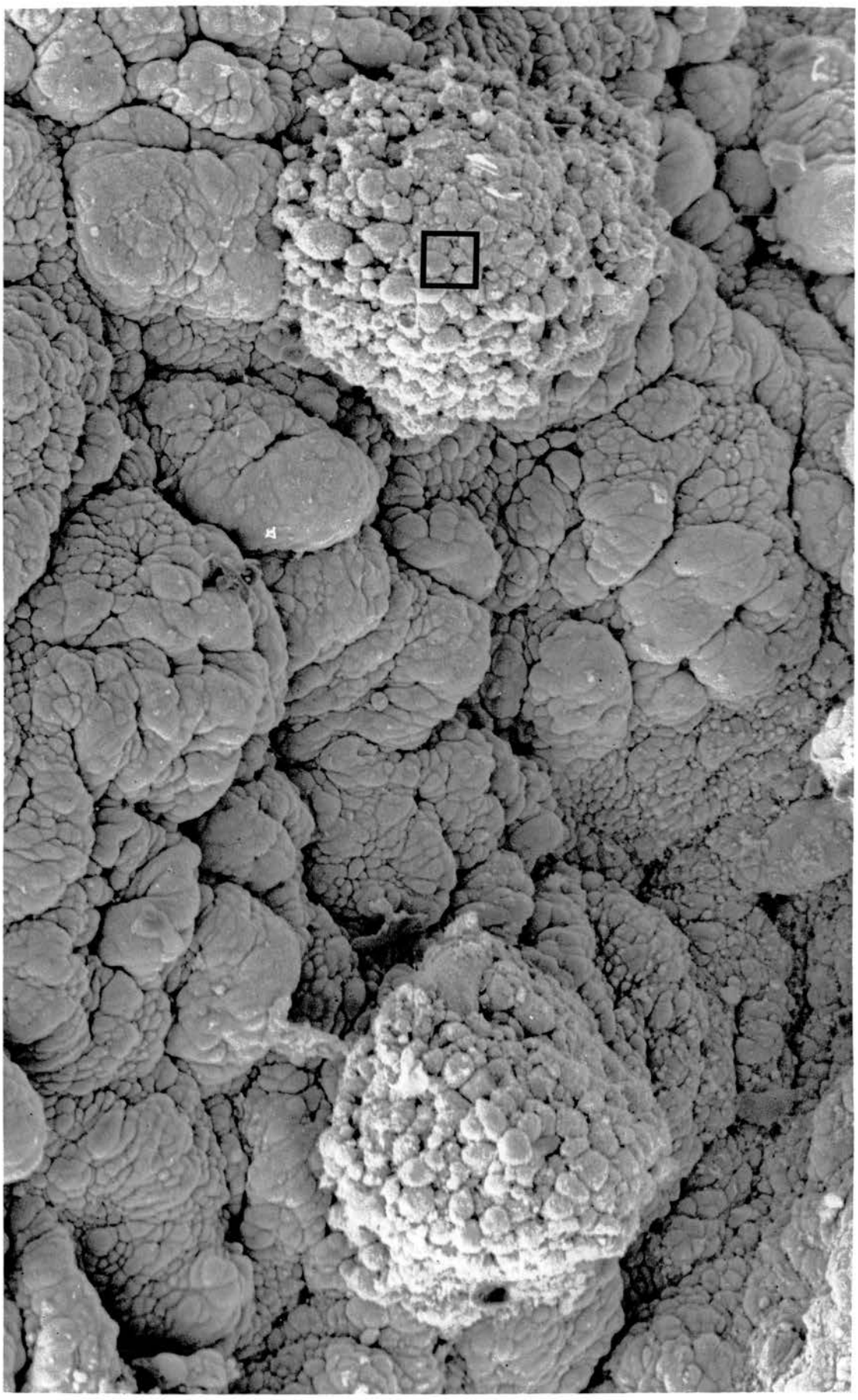
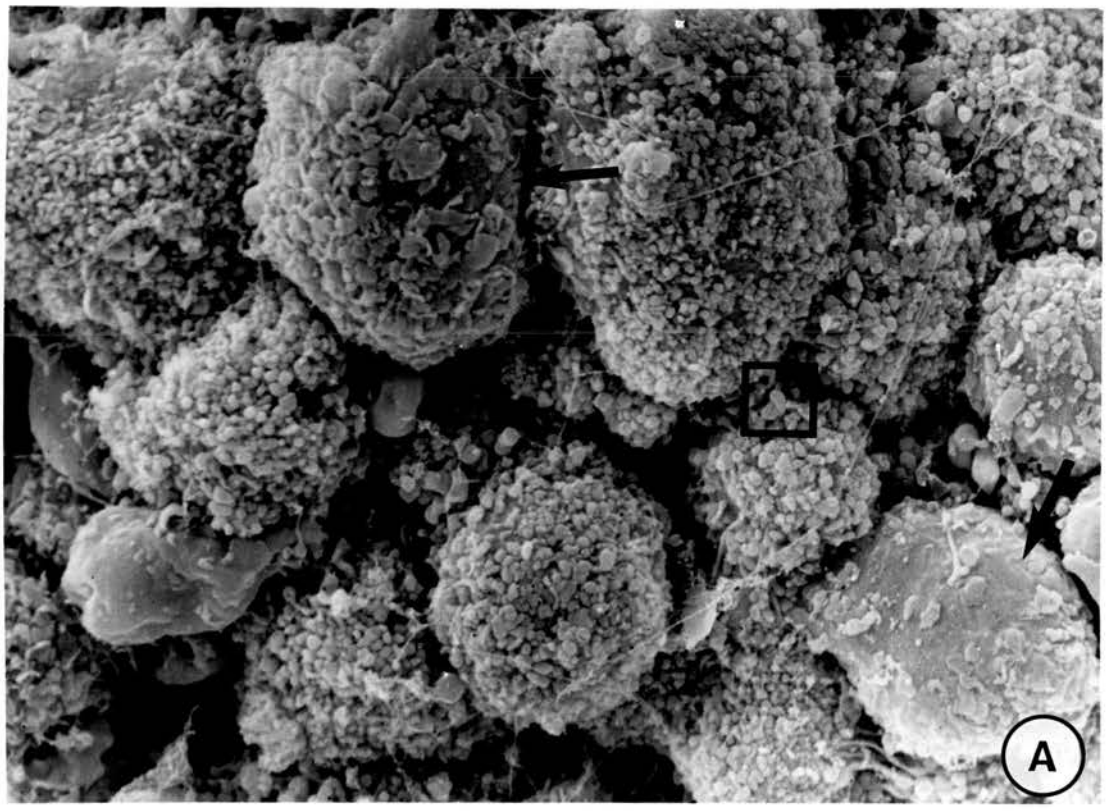
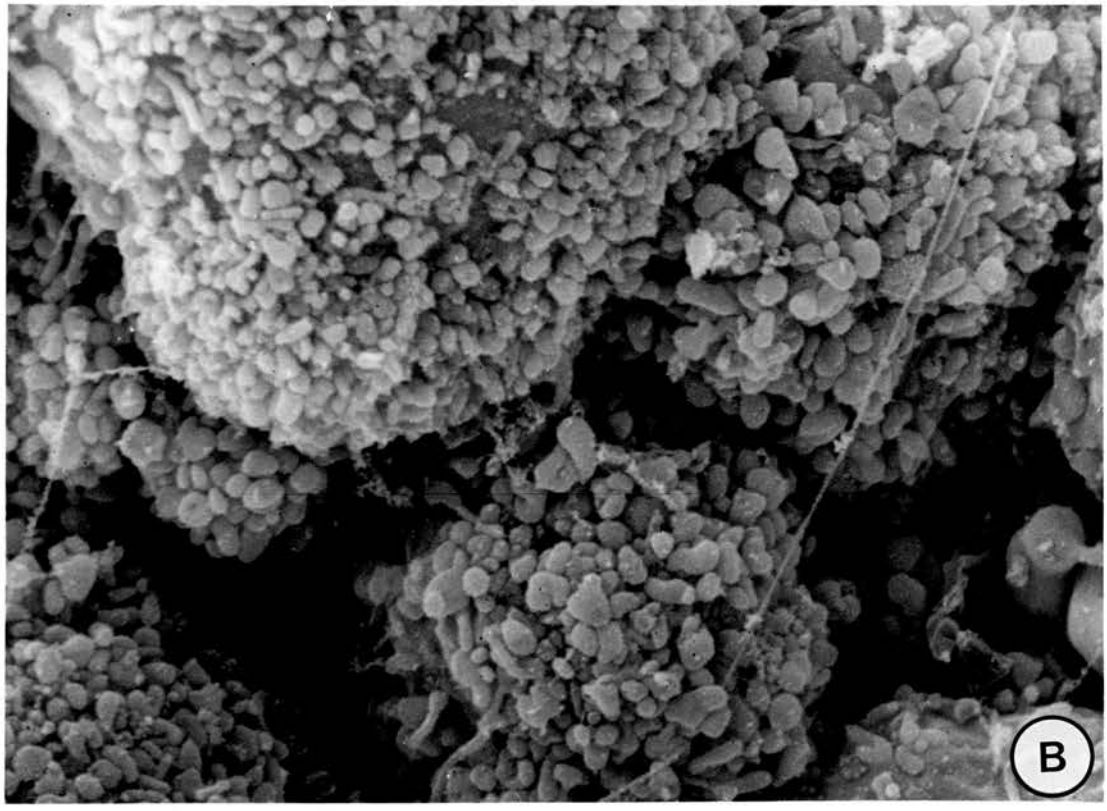


Fig. 6.14A,B

(A), higher magnification, (x 2500) of an area of cells marked by a square at the tip of the polyp shown in Fig. 6.13. Note the bizarre morphologies of the microvilli. Some cells show areas denuded of microvilli (arrows).

(B), Higher magnification, (x 6250) of an area marked by square in (A). Note blunt, irregular, or club-shaped microvilli are present.


A


B

Transmission electron microscopy:

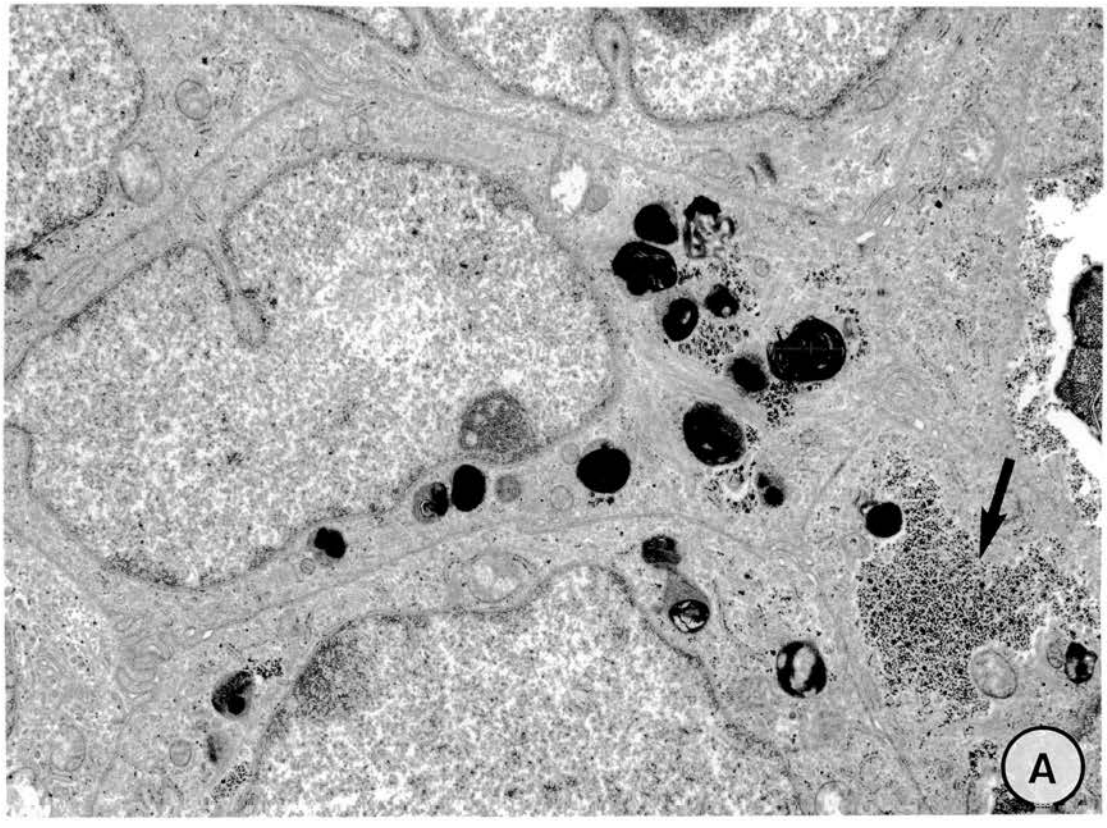
Transmission electron microscopy conducted on tumours induced in mice by cells of JS7, JS8 and JS15 showed that the tumour cells exhibited morphological and ultrastructural features characteristically associated with the parent cells (Fig. 6.15 & 6.16). They had desmosomes typical of epithelial cells (Picket et al, 1982) and microvilli were abundant and almost identical in structure to those of parent cells. Distinct bundles of tonofibrils were scattered throughout the cell cytoplasm and ribosomes were abundant. Glycogen was observed commonly, although the content varied considerably between individual cells. In some cells, large masses of glycogen granules were seen in the cytoplasm, whereas, in others there was much less and in a few, there was no evidence of glycogen at all. In many cells of tumours induced by JS7 or JS15, mitochondria were dilated and most had lost their cristae, but not in JS8 tumours.

Lamellar inclusion bodies were seen in varying sizes and number in many cells of tumours produced by JS7 and JS15 cell lines only. These structures were observed after 46 and 15 passages of cell lines JS7 and JS15 in vitro respectively. But lamellar bodies were not seen at any time in tumours induced by JS8 cell line (Fig. 6.16).

In one cyst produced by JS7 cells, serial transverse sections of a polyp were examined in order to determine the different cell types at various levels. It

Fig. 6.15A,B

Electron micrograph of nude mouse tumour cells induced by JS7, (A), and JS15, (B). Note the presence of glycogen (arrow) in (A) and Lamellar bodies in both sections (A, x 10000, B, x 8000).


A

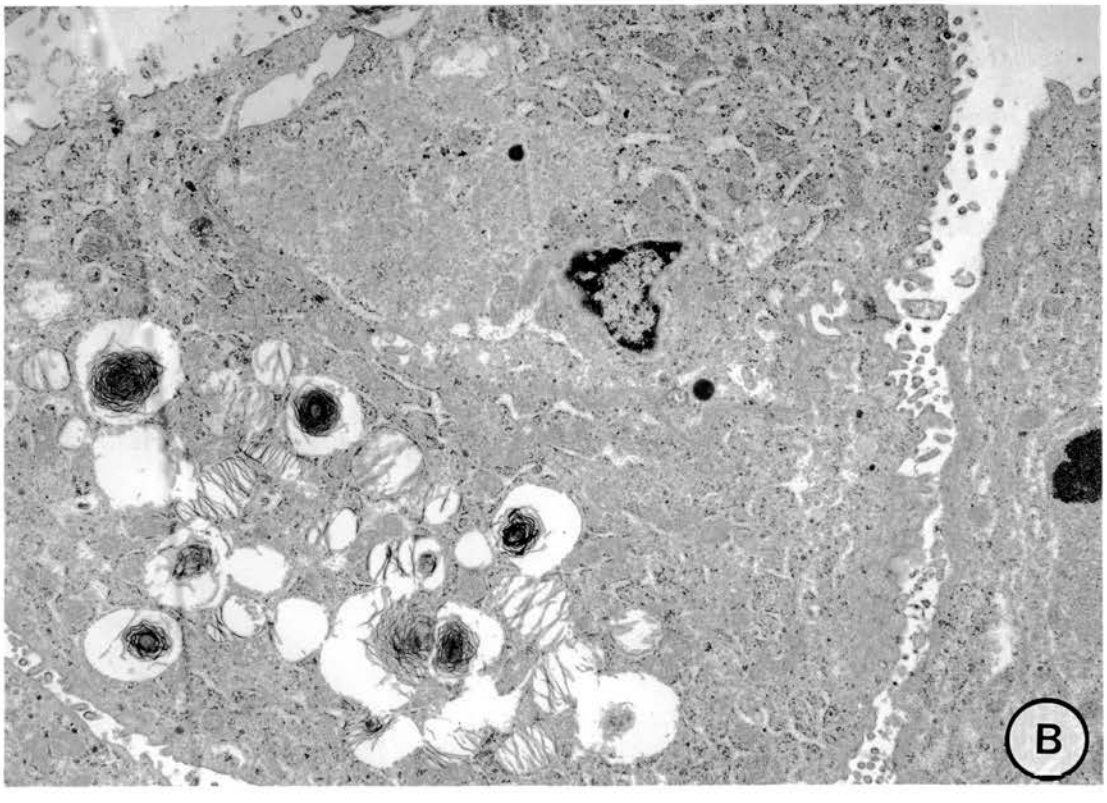
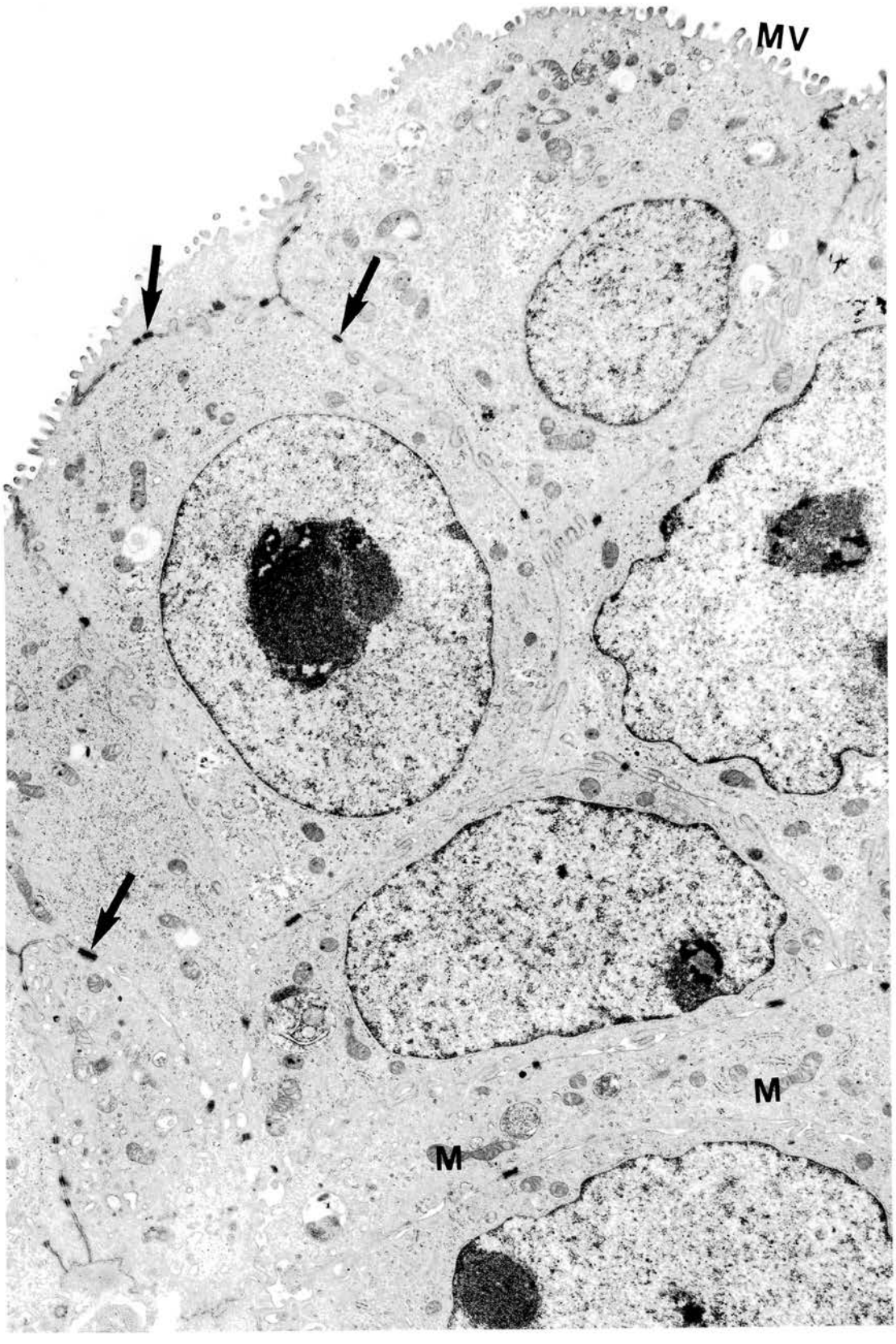

B

Fig. 6.16

Electron micrograph of JS8 tumour in nude mouse showing microvilli (Mv), numerous desmosomes (arrows); mitochondria (M); abundant ribosomes, but no lamellar bodies. x 9000.


MV
M
M

was found that the cells comprising the basal layer (Fig. 6.17A) were packed with lamellar bodies, and scattered or, more commonly, aggregates of glycogen granules. The nuclear membrane of these cells was very prominent, well-demarcated and displayed deep invaginations. Examination of serial sections moving up the polyp away from the lining epithelium and towards the free tip of the polyp showed a reduction in the number of lamellar bodies until, at the tip, no evidence of these was seen (Fig. 6.17B). However, glycogen granules were retained, with the nuclei assuming an oval or rounded shape. Some even showed pyknotic nuclei indicating necrosis.

In general the proportion of cells containing lamellar bodies and the number of lamellar bodies per cell was higher when compared with that of parent cells in culture.

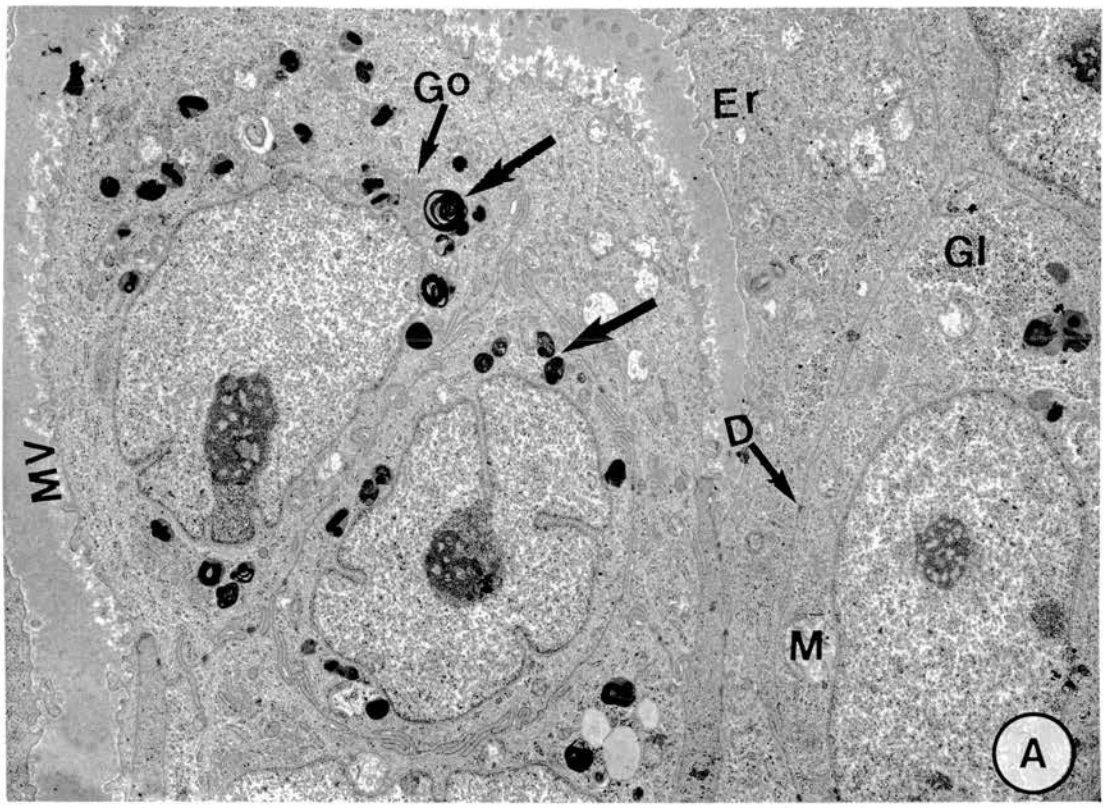
Virus particles, similar to type-C, were detected in the intercellular spaces or budding from the cell luminal surface in all samples obtained from tumours induced by the three cell lines (Fig. 6.18A). Additionally, particles 90 nm in size resembling type A retrovirus particles were detected in the cytoplasm or in dilated cisternae of some of these cells (Fig.6.18B).

Electron microscopy did not reveal virus particles in the cystic fluid by negative staining. Also the P25 of SPA viral protein was not detected in this cystic fluid by Western blot.

Fig. 6.17A,B

A), Cells of the basal layer in a cyst with several infoldings of nuclear membrane and interdigitation of plasma membrane. Cells have lamellar bodies (arrows); desmosomes (D) joining the cells to one another; numerous microvilli (Mv); Mitochondria (M) with lost cristae; well-developed golgi-apparatus (Go); glycogen aggragates (Gl); endoplasmic reticulum (Er) and ribosomes are present. (Electron micrograph, x 4000).

B), Cells at the tip of the same polyp in A showing, microvilli (Mv); desmosomes (small arrows); glycogen aggregates (large arrows); fewer ribosomes, but more mitochondria (M) with lost cristae. x 6000.


Go
Er
Gl
D
MV
M
A

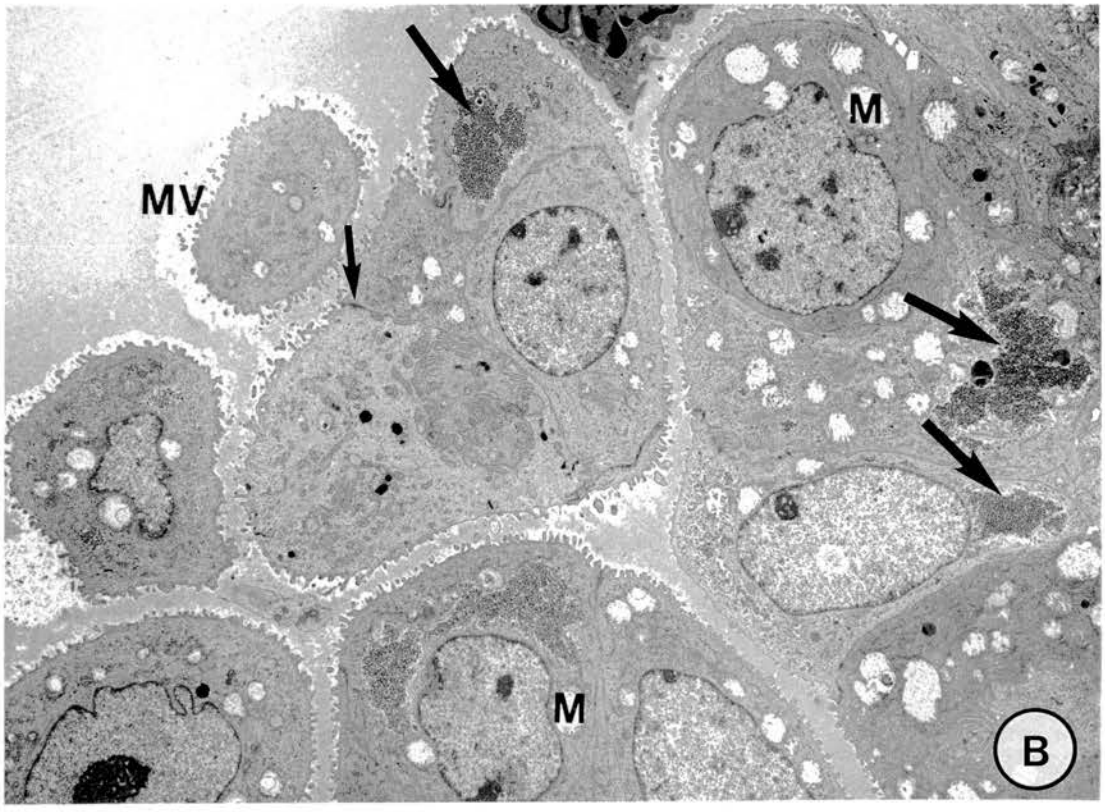
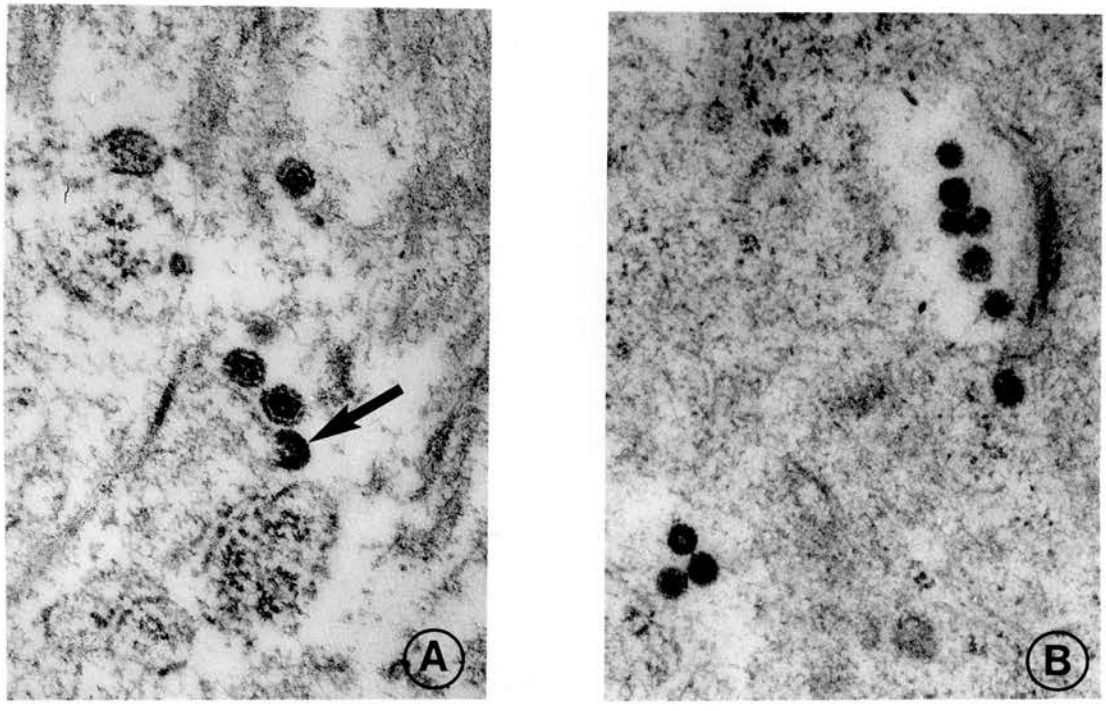

M
MV
M
B

Fig. 6.18A,B

A), part of a cell from a nude mouse tumour produced by JS15 cells, showing budding (arrow), and extracellular C-type virus particles. x 60000

B), part of a cell from a nude mice tumour induced by JS7 cells, showing clusters of intracisternal viral particles identical to A-type. x 45000


A
B

DISCUSSION

The most reliable marker of malignant transformation is tumour induction *in vivo* (Stiles *et al*, 1976). Following the transplantation in nude mice of cultured cell lines derived from the lungs of sheep with naturally-occurring SPA, cystic tumours filled with fluid developed at the site of injection. Similar tumours have been reported after transplantation into nude mice of minced SPA tumour (Zimber *et al*, 1984) or cultured cells (Verwoerd *et al*, 1977).

The size of the cystic tumours produced by SPA cell lines was accounted for mainly by the fluid contents. This observation indicates that the transplanted cells retain a secretory function. When considered in relation to the copious fluid produced in the lungs of sheep with jaagsiekte, it seems reasonable to assume that these SPA cell lines have retained functional activities similar to those demonstrated by the transformed cells in the natural host.

Cystic tumours engorged with fluid have been observed before. Clones of mouse mammary carcinoma were capable of inducing fluid-filled cystic tumours in which papillary structures extended into the cavity of the cyst and histological examination showed the tumour tissue to be undifferentiated (Sanford *et al*, 1961).

Similarly two human squamous cell carcinoma (SCC) cell lines derived from facial epidermis formed

large fluid-filled cystic tumours with histology characteristic of well-differentiated SCC (Rheinwald and Beckett, 1981).

In assessing the tumourigenic properties of JS7, JS8 and JS15 cell lines in nude mice, the results indicate no difference in the tumour-producing capacity of the three cell lines. However, differences in terms of final tumour volume were demonstrated in mice inoculated with the three different cell lines and even with the same cell line. This variation could be due to differences in susceptibility to transplantation among individual mice (Fogh et al, 1978) or to age or the genetic background of the mice.

The growth curve experiments have shown that after a latency period, the tumours induced by the SPA cell lines grew rapidly and ended with a plateau phase. This pattern of growth is in marked contrast to that which occurred with the control cell lines, A549 and NBL12, with which no plateau phase occurred. There appear to be several explanations for these two distinct types of growth although all involve the failure of the heterotransplants to become vascularised. It was clear that in the tumours induced by A549 and NBL12 there was intensive invasion by blood vessels, whereas, in the tumours produced by the SPA cell lines, no vessels could be observed. It is recognised widely that the growth of solid tumours is associated with the induction of new blood vessels. These vessels invade from the surrounding

host tissues due to the effects of tumour angiogenesis factor (TAF) secreted by the tumour cells (Folkman, 1974), and provide cell nutrients and remove waste products (Folkman, 1975). Thus the failure of the SPA tumours to develop a vascular supply would adequately explain the type of growth observed. However, it raises the question as to why no vascularisation occurred. Histological examination revealed that the SPA tumours induced in nude mice were enclosed by a fibrous capsule which could be demonstrated as early as seven days following the transplantation (the earliest time some of the tumours were examined histologically). Such a capsule could interfere with the growth of the tumours by affecting the vascularization of the transplants (Kriazis et al, 1978). Alternatively, the failure to vascularise could be explained by the inability of cells to secrete TAF and thus prevent further growth of tumour.

Thirdly, but less likely, the tumour may have developed rapidy, before angiogenesis, and due to the distension caused by the cystic fluid, inhibited the supply of blood thus depriving the tumour cells of nourishment.

In contrast to the present findings, Verwoerd et al (1977) reported numerous capillaries and some lymphatics in the epithelial layer of transplanted SPA. If this was a valid observation, it would be expected that the SPA tumours would show a growth pattern similar to A549 or NBL12 cell lines.

The latent period for tumour development ranged from 3-5 days with the four SPA cell lines used in this study, compared to 7 days or 14 days respectively for the observations of Verwoerd et al (1977) and Zimber et al (1984). The 14 day latent period seems unduly long compared with the present study and that of Verwoerd et al (1977). However, this variation might be because Zimber et al (1984) practised direct transplantation of minced tumour tissue rather than cultured cells as in this study or that of Verwoerd et al (1977).

Verwoerd et al (1977) have described in one of the two nude mice they used, the reestablishment of a progressive stage of tumour growth, following the stationary phase. In the progressive stage, the cyst increased in size until it ruptured. Because such a small number of mice was used, it is impossible to draw any conclusions from this report regarding the growth of SPA cells in nude mice. However, although the present study has produced conflicting results, on the basis of the quantitative and qualitative observations which involved the use of three different SPA cell lines and 41 nude mice in several experiments, it is suggested that the results of this study are more representative of the behaviour of SPA cells in nude mice. It was demonstrated that, for all SPA cell lines, the growth pattern was similar and consisted of a lag phase, followed by a log phase with rapid growth, and a plateau phase with no net increase

in tumour volume. The plateau phase was maintained until the tumour was excised between 6-7 weeks or even as long as three months after injection. Other workers have described plateau phases lasting as long as six months (Reid and Shin, 1978) or even, in some, cases the plateau phase remained until the mice were killed (Reid and Shin, 1978; Rheinwald and Beckett, 1981; Wilson *et al*, 1982).

The present investigations are incomplete because the time available for *in vivo* growth kinetic studies was limited to three months. A different growth curve might have occurred if the period of observation had been extended further since it has been shown for some human tumours that after a stationary phase of about four months, the tumour again starts to grow exponentially (Korsgaard *et al*, 1983). To take this fact into consideration, further growth kinetic studies would be necessary to clarify this and to draw a firm and convincing conclusion.

The tumourigenic ability of JS7 cells appeared to be dose-dependent. Although the number of mice in each group was small, it was clear that the minimum number of cells required to induce tumours in nude mice was not less than 10^6 cells. It has been suggested that the threshold number of cells needed to induce a tumour after the inoculation of cells in nude mice is a function of the specific cell injected (Freedman *et al*, 1976; Freedman and Shin, 1978). However, the observations of Norval *et*

al (1984) was that the number of cells required to induce tumours in nude mice with cell lines initiated from the same type of tumour varied. This would indicate that the data presented here for JS7 might not reflect the behaviour of cell lines initiated from other SPA tumours, therefore more cell lines need to be tested before judging how well the threshold number of cells correlate with tumour yield for indicating the degree of tumourigenicity.

Dependence of the rate of tumour initiation on the volume of inoculum also has been reported (Hirohashi et al, 1976; Freedman and Shin, 1978; Shouval et al, 1981).

Several investigators have described cellular and functional heterogeneity among the malignant cells isolated from individual primary tumours (Pierce et al, 1967; Pierce et al, 1977; Dexter et al, 1978; Becker et al, 1978). One expression of such heterogeneity is that subpopulations of such primary tumours and established cell lines can exhibit different in vivo tumourigenicity (Kimball and Brattain, 1980; Brattain et al, 1981; Miller et al, 1980). In a similar way the uncloned SPA cell lines used in the present studies may consist of subpopulations of low and high malignancy cells or even malignant and nonmalignant subpopulations as described for rat colon carcinomata (Caignard et al, 1985).

The presence of low malignancy or nonmalignant cells may not only decrease the effective number of cells injected but may also supress expression of malignancy of the high malignant cells as reported by Caignard et al (1985). It is clear from the wide range of karyotype that the SPA cell lines are heterogeneous (Chapter 4). However within each heterogeneous cell line, the presence of low and high malignant cells might be suggested by the observations in Chapter 4 that the subclones initiated from the parental JS7 cell line demonstrated different cloning efficiencies in soft agar, indicating that the cells of this cell line are heterogeneous in this particular character. It was also demonstrated that the capacity of this cell line to grow in soft agar, was dependent on the number of cells seeded (Chapter 4).

Despite prolonged in vitro passage of SPA cell lines, the tumours produced by these cells grew consistently as cystic tumours lined by epithelial cells. They maintained a consistent growth pattern and their constituent cells remained morphologically similar to the proliferating cells observed in the lungs of sheep with SPA. These observations indicate that the in vitro serial passage did not seem to impose selection pressure in the direction of abnormal variants or affect the in vitro phenotypic characteristics of SPA tumour cell lines. This is supported by the fact that none of the cystic tumours underwent change from a cystadenoma type to a mixed sarcoma/adenoma or pure sarcoma tumour as reported for

certain other tumours in mice (Sanford et al, 1961), or, as was the case in this study, in the A549 and NBL12 control cell lines.

A characteristic feature of all tumours induced in mice by SPA cell lines has been the formation of a fibrous capsule. The capsule may have resulted from stimulation of stromal cells by a substance liberated from SPA tumour cells. However, the role of this capsule in tumour development is not known. Possibly, it may provide adequate support and enhance the potential of these cells to develop the same characteristic pattern of frond-like or polypoid structures observed in SPA lung from which the cells were originated. Alternatively, the capsule may represent a non-immunologic defence mechanism of the host.

Cells covering the tip of the polyps, showed evidence of necrosis with bizarre microvilli. Cell necrosis could have been due to pressure resulting from the increasing size of the proliferating cell mass in the polyp, or because of the residence of these cells in a physiologically unfavourable environment, in which the nutrients or oxygen supply did not meet the metabolic demands of the cells, or some combination of these and other factors.

Ultrastructural studies showed that the cells of the cystic tumours were epithelial in morphology and that the heterogeneity of cells of sheep lung with pulmonary adenomatosis (Perk et al, 1971; Nisbet et al, 1971; Angus et al, 1985) was maintained in cell line and

in mouse tumours. These observations provide further evidence for the stability of the cell lines, and that no selective growth advantage occurred *in vitro*.

Lamellar bodies were demonstrated in nude mouse tumours induced by JS7 and JS15 cell lines only. The presence of these lamellar bodies in transplants and their corresponding cell lines contradicts the report of Stoner *et al* (1975) who observed that a tumour produced by cloned LA-4 cells (mouse lung adenoma) did not contain lamellar bodies although other features of this cell line indicated that it was a derivative of type II alveolar pneumocytes.

The cells of JS8 did not show the one ultrastructural feature characteristic of differentiated type II pneumocytes, namely, the occurence of lamellar bodies. However, despite this finding, fluid still accumulated in the tumour induced by these cells, but not as much as was elicited by the other two SPA cell lines (JS7 and JS15). The small proportion of type II pneumocytes demonstrated by Phosphine 3R in the cell suspension (Table 6.1) could explain why there was less fluid in the mouse tumour induced by the JS8 cell lines.

In tumours induced by JS7 and JS15 cell lines, type-C particles were seen within the intercellular spaces or in the process of budding from the cell surface. In addition, intracytoplasmic and intracisternal A particles were also found.

It has been reported that tumour cells transplanted into nude mice, have a high possibility of picking up murine retroviruses (Kuga et al, 1975; Price et al, 1975; Achong et al, 1976; Suzuki et al, 1977; Friend et al, 1978; Crawford et al, 1979; Wunderli et al, 1979; Gautsch et al, 1980; Beattie et al, 1982). In these studies the type-C viruses found in transplanted tumours were regarded as murine endogenous viruses of nude mice origin because type-C particles have never been detected in the SPA cell lines grown in vitro nor in SPA tumours taken directly from affected sheep in Scotland.

The presence of type A retrovirus particles in tumours induced by JS7, JS15 and JS8 cells is not without precedent, as similar particles were detected in experimentally induced pulmonary adenoma in BALB/C mice by by Bucciarelli and Ribacchi (1972).

Intracytoplasmic type A retrovirus particles have been found in experimentally induced tumours in young lambs (Sharp et al, 1983) and also in experimentally induced jaagsiekte in young lambs with SPA cell lines (Chapter 8). No evidence of viral infection of JS15 and JS7 cell lines could be demonstrated by Western blotting beyond the 4nd and 11th passage respectively. However, the possibility that JS7 and JS15 cells carrying very small numbers of the transforming virus grew selectively in nude mice cannot be excluded.

CONCLUSIONS

1. Transplantation studies show that the JS7, JS8, and JS15 cell lines initiated from SPA tumour are tumorigenic. They can be heterotransplanted to athymic mice which may be used as a host for SPA tumours.

2. The histological and histochemical features of the tumour induced in mice by SPA cell lines at different passage levels resembled that of SPA tumours in sheep. Growth *in vitro* for extended periods, in particular of JS7 and JS8, did not alter the ability of these cell lines to produce tumours with the same histological characteristics.

3. Lines JS7, JS8 and JS15 induced tumours that demonstrate several ultrastructural features in common with that of the original tumour from which they were derived.

4. The SPA cell lines maintain an ability to secrete fluid in nude mice.

5. Finally these tumour cell lines which retain differentiated characteristics, *in vivo* and *in vitro*, may prove valuable to those interested in investigating the process of differentiation and, in particular, the proper interpretation of the factors involved in the production of the copious exudate in the lungs of some sheep affected with pulmonary adenomatosis and the production of cystic rather than solid tumours in athymic mice.

CHAPTER 7

CHARACTERISTICS OF SPA CELL LINES FOLLOWING PASSAGE IN NUDE MICE

INTRODUCTION

The major interest in studying the characteristics of the cells obtained from the tumours after transplantation was three-fold. First, to see whether the cultivated cells had retained the features of the parent cell lines; secondly, to determine whether passage through nude mice resulted in selective growth advantage for a subpopulation of the parent cells; and thirdly, to show that the tumours were derived from the introduced cells rather than by proliferation of mouse cells.

To take these facts into account it was necessary to attempt to culture the nude mouse tumours and to establish cell lines for analysis.

In this chapter, cultures were initiated from nude mouse tumours resulting from heterotransplantation of JS7, passage 46 and JS8, passage 52. The cell cultures derived from the transplants were designated JS7(M)* and JS8(M). These two cell lines were studied in terms of growth as monolayer cultures, chromosomal analysis, ultrastructural characteristics and colony formation in semisolid medium. However, ultrastructural characteristics

* denotes, mouse

and growth in semisolid medium were not obtained for JS8(M) cells because, at this stage of study, the nude mouse tumour from which this cell line was initiated was found to harbour an endogenous murine retrovirus. As a precaution to protect the parent SPA cell lines from becoming contaminated with the murine virus, no further studies were conducted on either JS7(M) or JS8(M).

Materials and Methods

Cell culture

Initiation of cultures.

It was shown in the preceding Chapter, that the transplanted cells induced in nude mice cystic tumours filled with fluid containing free cells. The cysts were lined by epithelial cells, from which papilliform ingrowths arose at intervals. Therefore, cultures were initiated from free cells as well as those lining the cyst.

a). Initiation of cultures from free cells

Cystic fluid was aspirated aseptically and centrifuged at 200 xg for 5 minutes at 4°C. The pellet was then washed twice with 10 ml fresh growth medium to remove debris before resuspending it in 5 ml of growth medium. The entire cell yield of one cyst was then seeded into one 25 cm^2 plastic culture flask and incubated at 37°C, with the stoppers kept loose, in a humidified atmosphere of 5% CO_2 in air. The cultures were left for 48 hours without being disturbed to permit adherence and initial growth.

In the primary cell cultures, after 48 hours of incubation, dead cells and debris were removed by aspirating the medium without disturbing the adherent cells and then refeeding with 5 ml of medium. From then on, all flasks were fed every 48 hours by complete removal of the spent medium and replacement with 5 ml of fresh medium.

b). Initiation of cultures from the lining cells

The walls of the cysts, after aspirating the contents, were removed aseptically from mice and placed in a sterile 25-ml beaker containing 10 ml of phosphate buffer saline at 37°C. In this beaker the tumour was minced finely with scissors. The fine mince was then washed in fresh phosphate buffer saline and a suspension of single cells obtained by 2 x 20 minutes incubation with 25 ml of 0.25% trypsin solution at room temperature. After trypsinization, the cells were collected by centrifugation at 200 xg for 5 minutes at 4°C. The sedimented cells were resuspended in 5 ml of growth medium and then were treated in a manner similar to that described in (a).

Growth in soft agar

A single passage of JS7 cells through nude mice may have resulted in a selective growth advantage that would be reflected by improved growth in soft agar. Also the cloning efficiency of JS7 cells, before inoculation, with that of JS7(M) was compared.

Cells were suspended in soft agar medium at a density of 10^5 cells per plate as described in General Materials and Methods.

Karyology

Chromosomal analyses were conducted on exponentially growing monolayer cultures seeded in 75 cm^2 plastic flasks by incubating the cells with colchicine (0.05 µg/ml) for 5 hours. Cells were then harvested and processed as described in General Materials and Methods.

Electron microscopy

Cultured cells were fixed in 1% buffered glutaraldeyde for 10 minutes, harvested by scraping, and processed as shown under General Materials and Methods.

RESULTS

Morphology and growth characteristics

Whether initiated from free cells or cells lining the cyst, small islands of pure epithelial cells without contaminating fibroblasts arose within 24 hours of plating. Uniform confluent monolayers of epithelial cells developed from these islands in another 4-6 days. Cultures initiated from free cells or cells lining the cyst were morphologically similar and indistiguishable from their respective parents with which the mice had been injected (Fig. 7.1). Both JS7(M) and JS8(M) cells were cultured for prolonged periods without any apparent decrease in their growth rate, and multiplied well even when plated at an extremely low cell density of 22 cells per cm^2. This was the lowest cell density attempted, and at which JS7(M), JS8(M) and their respective parent cells were found to grow.

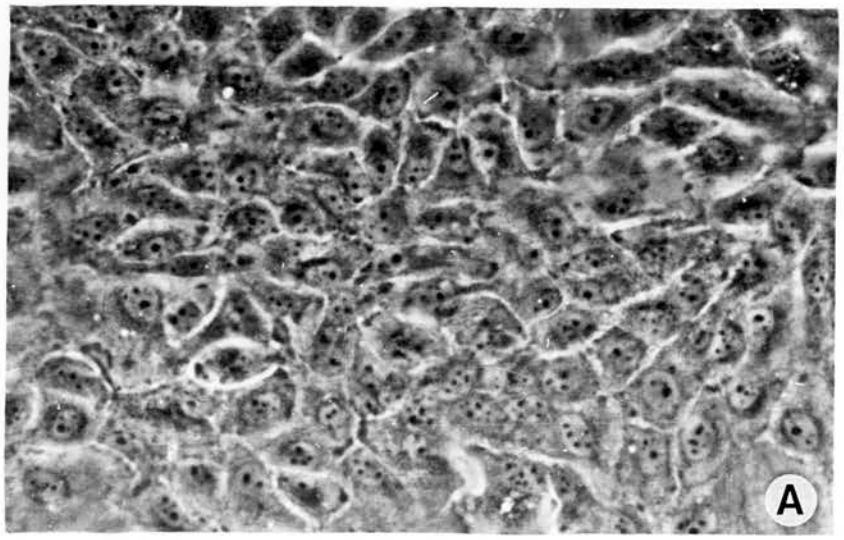
Growth in soft agar

Results presented in Table 7.1 show that cells of JS7(M) grew well in soft agar with a cloning efficiency of 7.5% which is significantly ($P < 0.001$) higher than the parent cells. The size of the colonies formed with JS7(M) was similar to those formed by the parent cells. However, JS7(M) displayed less variation in the morphology of the colonies.

Fig. 7.1A,B

A) Shows JS7(M) cells on day 4 in monolayer culture, and it depicts a cobble-stone appearance, which is characteristic of the parent cells, Phase contrast. x 205

B) Cells of JS7(M), on 7th day after plating showing loss of contact inhibition. Light microscope. x 102


A

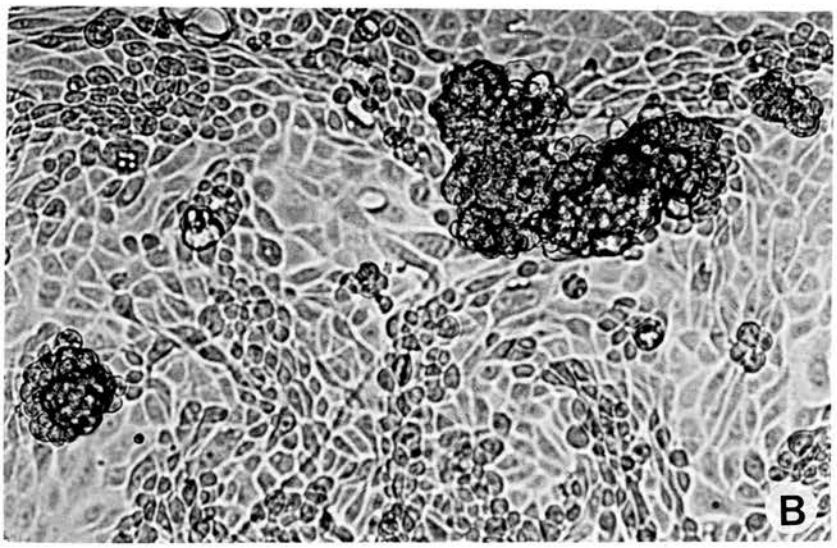
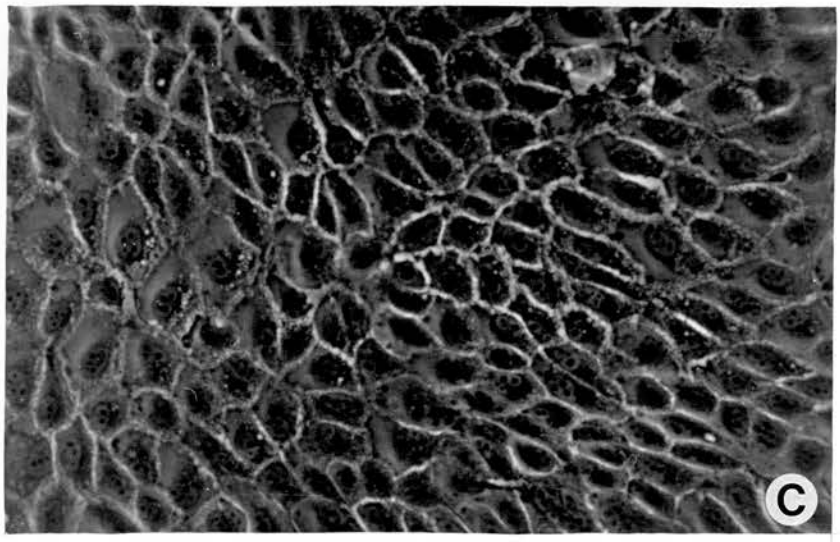

B

Fig. 7.1C,D

C) Epithelial cell culture of JS8(M) cells 4 days after plating. Cells are polygonal in morphology. Phase contrast. x 205

D) Cells of JS8(M), on 7th day after plating showing loss of contact inhibition. Light microscope. x 102


C

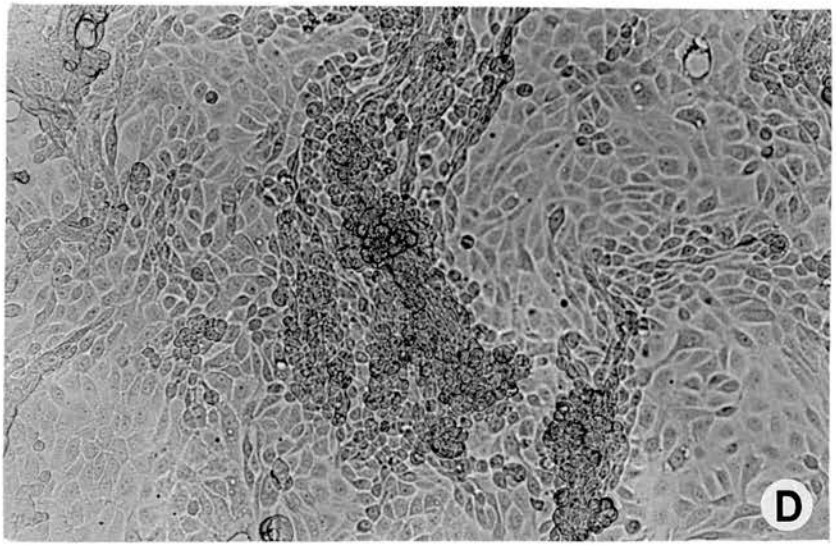

D

Table 7.1

Cloning efficiency of JS7 cells in semisolid medium

Cell line	Number of colonies/dish	% cloning efficiency
JS7	4163±77	4.2
JS7(M)	7495±72	7.5

One hundred thousand cells of JS7(M) after three *in vitro* passes, were plated in soft agar as described in "General Materials and Methods". Colonies were counted after 14 days of plating. Values represent mean of colonies in three plates.

Karyological analysis

Chromosome counts of JS7(M) and JS8(M) cells were made at the 2nd passage after they had been initiated from nude mice tumours. Sixty-six metaphase spreads of each cell line were examined. As shown in Fig. 7.2, cells of JS7(M) and JS8(M) showed a wide range in chromosome number and had a modal number of 74 and 68-69 respectively (Fig. 7.3). The analysis demonstrated large metacentric chromosomes in all metaphases. This is characteristic of the normal ovine karyotype which consists of 6 large metacentric and 46 acrocentric or telocentric chromosomes (Fig. 7.4), and no spreads consisting of mouse chromosomes were seen.

Of the sixty-six metaphases, 27 and 41 metaphases of JS7(M) and JS8(M) were selected respectively to study the structure of each metaphase. This number of metaphases was selected because they were well spread and easy to analyse. The results are shown in Table 7.2 and the appendix 3.

The lines contained submetacentric chromosomes, shown in all metaphases. Dicentric and long submetacentric chromosomes were demonstrated in very few cells. These unsual chromosomes were also seen in the parent cell lines as shown in Chapter 4.

Fig. 7.2

Histogram depicting the distribution of the number of chromosomes per cell for 66 metaphases of JS7(M) and JS8(M) cell lines.

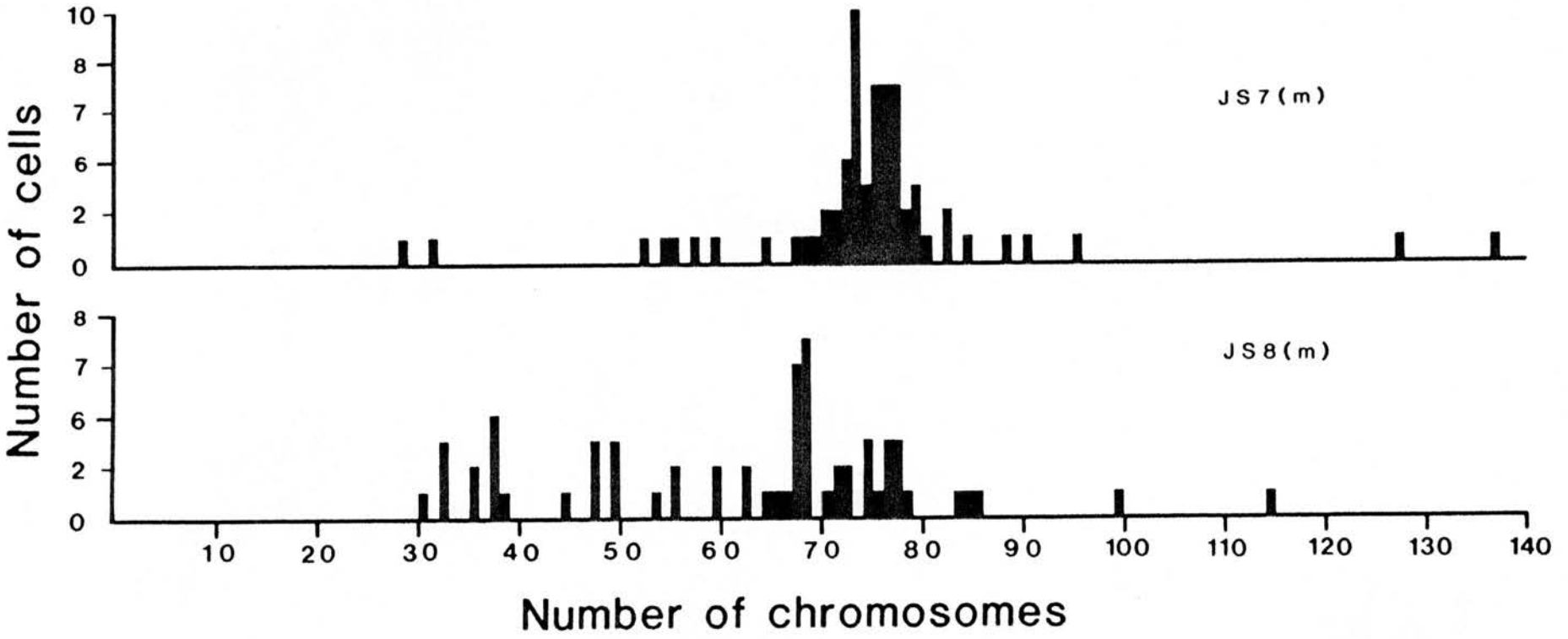

Number of cells
10
8
7
6
2
0
JS7(m)
8
7
6
2
0
JS8(m)
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Number of chromosomes

Fig. 7.3A,B

Metaphases in mouse-passaged SPA cells. A) JS7(M); 74 chromosomes with submetacentrics (arrows). B) JS8(M); 68 chromosomes. The arrow indicates a dicentric chromosome, and the inset shows a long submetacentric chromosome.


A


B

Fig. 7.4

Metaphase, showing normal diploid ovine chromosomes (2n=54).

Table 7.2

Structure of metaphases in JS7(M) and JS8(M) cell lines

Chromosomal regions	JS7(M)	JS7*	JS8(M)	JS8*
2n	3(4.6)	1(1.52)	22(33.3)	3(4.6)
3n	55(83.3)	10(15.2)	38(57.6)	9(13.64)
4n	6(9.1)	53(80.3)	5(7.6)	50(75.8)
5n	1(1.52)	-	1(1.52)	1(1.52)
6n	1(1.52)	-	-	-
7n	-	2(3.03)	-	3(4.6)

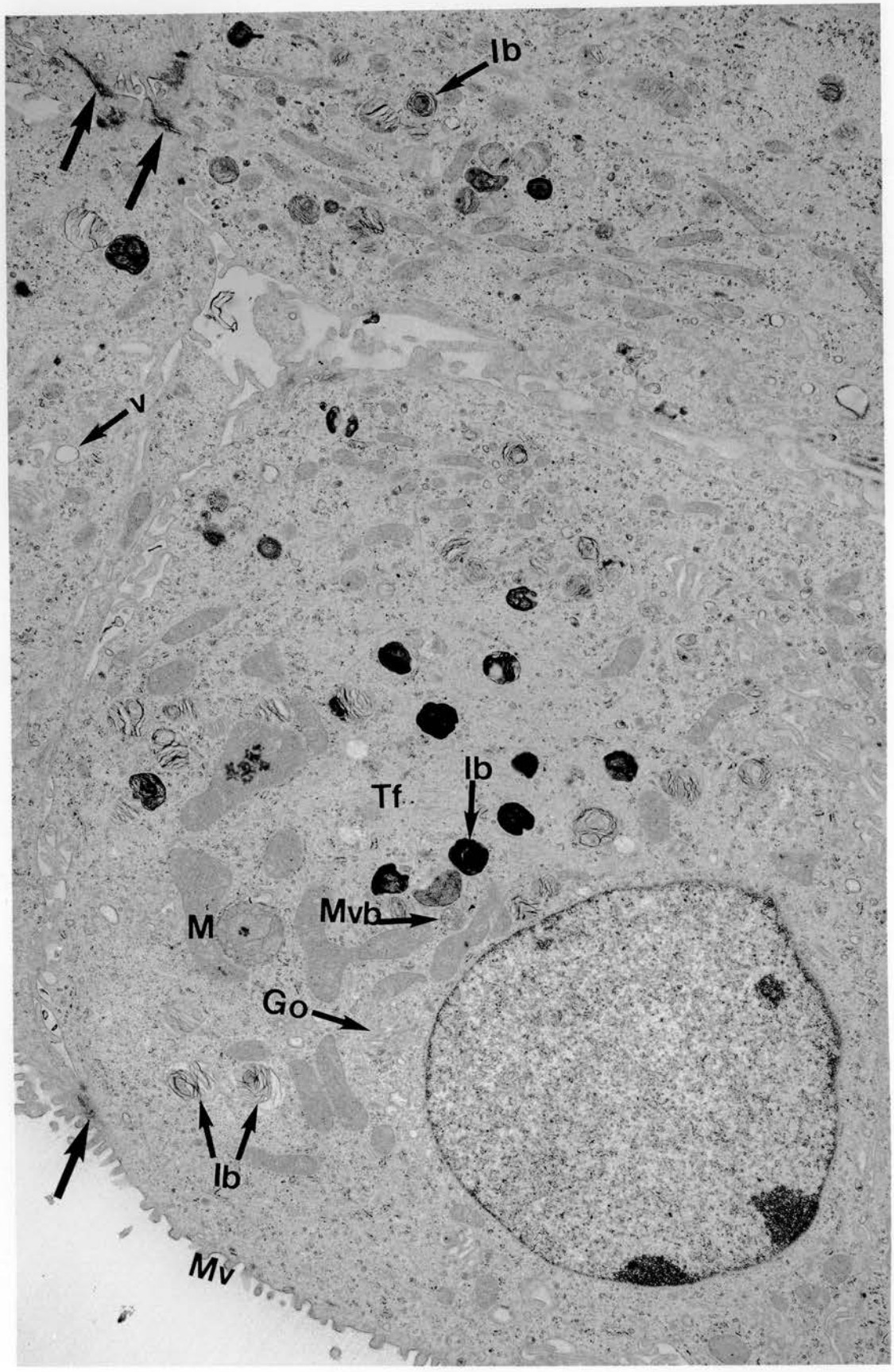
*
karotype conducted on passage 20.
n = 27 chromosomes.
Figures in parentheses denote percentages.

Ultrastructural features

Electron microscopic examination confirmed the light microscope findings that JS7(M) cells had retained features of the original JS7 parent cells and the nude mice tumours from which they were derived. The cells exhibited epithelial morphology as evidenced by desmosomes and microvilli (Fig. 7.5). The cytoplasm contained lamellar bodies, multivesicular bodies, vacuoles, Golgi apparatus, abundant ribosomes and endoplasmic reticulum. Small amounts of indistinct glycogen granules were present as were well preserved mitochondria, none of which had lost their cristae as seen in nude mouse tumour biopsy. No virus particles could be detected in these cells.

Fig. 7.5

Transmission electron micrograph of cultured JS7(M) cells. A number of desmosomes (arrows) formed between cells can be seen. The apical surface is covered by microvilli (Mv). In the cell cytoplasm, lamellar bodies (Lb); well-developed mitochondria (M); Golgi apparatus (Go); bundles of tonofilaments (Tf); free ribosomes; and membrane bound vacuoles (V) can be seen. Multivesicular bodies (Mvb) are evident. x 9000.


lb
v
Tf
lb
M
Mvb
Go
lb
Mv

DISCUSSION

The studies conducted on JS7(M) and JS8(M) confirm that the mouse-passaged tumours consisted of ovine cells. This is apparent from karyological analysis of these cells which demonstrated ovine chromosomes in all metaphases examined. The results obtained indicate that cell cultures established from heterotransplanted tumours might be useful for *in vitro* assessment of the malignant SPA epithelial cells. These cultures grew well without contaminating fibroblasts and could be propagated continuously. The cells were morphologically and ultrastructurally similar to the parent cell lines or SPA lung tumour in sheep.

It has been shown in this study, that the transplanted tumour can be readily explanted into tissue culture and serially passaged. This is in contrast to the report by Verwoerd *et al* (1977), that cell culture for mass production cannot be established from SPA tumours grown in nude mice. The cells used by these workers for transplantation was not a permanently established cell line, which may account for their conflicting results.

Cytogenetic studies showed that deviations from parent cells are found in the chromosome constitution of both JS7(M) and JS8(M) cells. In particular, the cells with a triploid chromosome number predominated as compared to both parent cell lines in which the tetraploid cells were dominant. This may reflect

an in vivo selection pressure on the tetraploid cells and selective growth of the triploid during tumour development. However, another explanation is possible. The chromosome analysis of the JS7 and JS8 cells was conducted at the 20th passage whereas JS7(M) and JS8(M) cells were initiated from tumour induced by the 46th and 52nd passage respectively, of JS7 and JS8 cells. It is possible that the observed chromosomal variation may have taken place during the intervening 26-32 passages of parent cells before inoculation into nude mice. Therefore, the modal number reported here may be a result of in vitro changes and does not reflect any event in the nude mice. Such changes are not unexpected, since other investigators have shown that cells grown in vitro undergo change in the number or structure of chromosomes, which results either in an increase (Ferguson and Tomkins, 1964) or decrease in the modal numbers (Hill et al, 1970; Poiley et al, 1978).

JS7(M) cells when grown in semisolid medium displayed a cloning efficiency higher than the parent cells. Growth in soft agar is a reliable criterion in assessing the neoplastic state of cells grown in vitro (Stiles et al, 1976). Therefore, derivation of JS7(M) cells with higher anchorage-independence suggests that there may have been selection in the nude mouse in the favour of more malignant cells.

Virus particles were observed in nude mice transplants. In contrast, no virus particles were

detected in the epithelial tumour cells from nude mice when grown *in vitro*. The absence of virus particles in nude mouse tumour cells conforms well with the failure to demonstrate virus particles in cell lines derived from lung tumours of sheep when propagated continuously *in vitro*.

CONCLUSIONS

1. The heterotransplant is of sheep origin. This is indicated by retention of the morphological and ultrastructural features together with the existence of sheep chromosomes in all metaphases and the absence of mouse chromosomes in cells reestablished in culture from tumours grown in nude mice.

2. Growth of JS7(M) cells in soft agar with cloning efficiency higher than the parent cells indicates that selection in favour of highly malignant cells had occurred.

CHAPTER 8

TRANSMISSION OF SPA BY MEANS OF EARLY PASS OR PERMANENTLY ESTABLISHED LINES OF SPA CELLS

INTRODUCTION

It has been well documented that SPA can be transmitted experimentally by preparations containing SPARV (Sharp *et al*, 1983; Verwoerd *et al*, 1983; McCullough *et al*, 1985; DeMartini *et al*, 1987), but less well documented with tumour cells or inocula arising from such cells (Verwoerd *et al*, 1980a). In addition, no tumour induction in lambs has been reported by SPA cell lines that have been permanently established *in vitro*.

Coetzee *et al* (1976) reported the induction of jaagsiekte in three neonatal lambs after inoculation of 24th passage of SPA tumour cell culture followed by immunosuppressive therapy. Transmission of the disease in newborn lambs was also achieved by inoculation of a microsomal fraction of a cytoplasmic extract of cultured tumour cells or tumour tissue (Verwoerd *et al*, 1980b). The cell line used by these workers was karyologically male, although it was derived from a female donor. Cytogenetic studies conducted on tumours induced by this cell line showed they were of the same sex as the recipient sheep. Despite these results, the authors suggested that their findings indicated that the tumour resulted from proliferation of the inoculated cells, i.e.

transplantation had occurred. In the present study, two SPA cultures, JS7 and JS8, have been established as permanent cell lines and virus replication was demonstrated in these cultures during early passages.

This chapter describes the successful transmission of SPA to neonatal lambs by permanently established cell lines. Furthermore, evidence is presented for the presence of SPARV in the induced tumour and also cell cultures derived from these tumours.

MATERIALS AND METHODS

Lambs

Lambs at the age of 2-48 hours were obtained from flocks maintained at the Moredun Research Institute. The lambs were of different local breeds (Suffolk, Blackface, Greyface, Dorset). Lambs were either left with their mothers or separated after birth and hand fed.

Cell lines

In this study cell lines JS7 and JS8, grown as described in Chapter three, were used.

Experimental design

Oncogenicity of the SPA cell lines was assessed in three separate experiments.

Experiment 1

In this experiment, cells of JS7 (passage 24) and JS8 (passage 16) were inoculated intratracheally at varying concentrations into 8 lambs (Table 8.1 and Table 8.2).

Lambs inoculated with one particular cell line were kept together in boxes separated from other groups of lambs.

All lambs were housed in their original boxes throughout the experiment which was terminated at six to eight months post-inoculation.

Experiment 2

Since it was important to determine whether SPA induction in lambs resulted from transplantation of the inoculated cells or from transformation of the recipient's own lung cells, groups of 3 and 4 lambs were given cell homogenate or concentrated culture medium respectively of JS8 cell line at the same passage level as above. The cell homogenate was prepared by homogenising JS8 cells at passage level 16, for 15 minutes using a MSE homogenizer (serial number, 770103). During homogenization, cells were kept surrounded by ice in a beaker, then made up to 5 ml volume in medium containing 10% fetal bovine serum. The 5 ml volume was adjusted to contain the equivalent homogenate of 10^8 cells. Before use the disrupted cells were kept at -70°C, then thawed once to ensure no viable cells remained. Cells were also examined by light microscopy and the trypan blue test to determine their integrity and viability. The concentrated culture medium from JS8, passage 16, was prepared from confluent cultures (cells seeded at the density to reach confluency in 2 days) and, 48 hours later, culture medium was collected and processed in the same way as lung fluid (General Materials and Methods). Lambs receiving intact cells in experiment 1 served as positive controls for experiment 2, because both experiments were conducted at the same time.

Experiment 3

This experiment was carried out to determine whether the permanent cell lines maintain their oncogenicity after long-term cultivation in vitro. Material for inoculation was obtained from cultures of JS7 (passage 137) and JS8 (passage 125). In this experiment, 8 lambs were used for JS7 and 5 lambs for JS8. Lambs were inoculated intra-tracheally with 30-120 x 10^7 cells in 5ml of growth medium as indicated in Table 8.4 and 8.5.

Necropsy procedures

Animals were killed with pentobarbitone. Before opening the thoracic cavity, the trachea was exposed and clamped with forceps to prevent lung collapse. The lungs were removed and inspected carefully. If lesions were seen they were excised and fixed as described in "General Materials and Methods". In cases where no lesions were seen, the lungs were fixed by perfusing the fixative through a needle inserted in the trachea. After the lungs were filled with fixative they were immersed in further fixative for 48 hours. Fixed lungs were serially sliced into 0.5 cm thick slices and both sides of each slice were inspected for the presence of lesions. All suspicious foci were excised and processed to paraffin-wax. Additionally, at least 18 blocks from preselected slices in the dorsal and ventral aspects of each lung lobe were processed similarly.

Electron microscopy

Immediately after removal of the lungs, small pieces of lesions were cut and fixed in 3.2% glutaraldehyde and processed as described in "General Materials and Methods".

Chromosome studies

To demonstrate that the tumours induced in lambs by JS7 and JS8 cells were virus induced and not transplanted SPA cells, karyotype analysis of the SPA cells and those of tumours induced in recipient lambs was performed. Because JS7 and JS8 cells were of female karyotype, primary cultures raised from male lambs which showed typical lesions of SPA were examined. The sex and chromosomal alterations of these cultures were determined as described in General Materials and Methods.

Blotting

To find out whether SPARV could be detected in cultures derived from lungs with SPA lesions or lung fluid collected from affected lambs, the immunoblotting technique described in General Materials and Methods was used.

RESULTS

Oncogenicity of epithelial cells cultured from SPA tumours

Cells of JS7 and JS8 were found to be oncogenic when inoculated into new born lambs.

In experiment 1, (Table 8.1), two (25%) of the lambs, which received JS8 cells, developed clinical signs of pulmonary adenomatosis after 150 and 179 days respectively. The signs of illness were dyspnoea, moist rales and abdominal lifting. When the affected lambs were tipped up, 30 ml lung fluid was obtained from one. A further lamb killed at 216 days post-inoculation had only microscopic lesions of SPA in the lungs. None of the remaining 5 lambs inoculated with this cell line developed lesions.

Four of the 8 lambs inoculated with JS7 cells (Table 8.2) developed typical signs of pulmonary adenomatosis. Signs were first evident in one lamb at 147 days, after injection another lamb at 158 days, and in two lambs at 170 days. Fifteen to 20 ml lung fluid was collected from two of the lambs.

In experiment 2, (Table 8.3) cell homogenates induced SPA lesions in one of the three inoculated lambs. Lambs inoculated with cell free tissue culture fluids from JS8 failed to show any signs of the disease or lesions.

In experiment 3, lambs did not show clinical signs of SPA whether inoculated with JS7 or JS8 cells.

Table 8.1

Oncogenicity of JS8 cell line (passage 16) in neonatal lambs

Lamb number	Breed	Sex	Age (hrs)	Number of cells* inoculated	Tumour development
205	S	M	5	10.5	-ve
1552	D	F	2	8	-ve
1564	S	F	4	8	-ve
1553	D	M	5	6	+ve
203	S	M	7	5.3	+ve
204	S	M	7	5.3	-ve
427	S	F	24	4	-ve
436	S	M	4	3.3	+ve

* $\times 10^7$ cells

(M), male; (F), female; (S), Suffolk; (D), Dorset.

Table 8.2

Oncogenicity of cell line JS7 (passage 24) in neonatal lambs

Lamb number	Breed	Sex	Age (hrs)	Number of cells* inoculated	Tumour development
230	B	F	2	12.5	-ve
229	B	M	2	12.5	+ve
1554	D	F	5	10	-ve
232	B	F	7	10	+ve
231	B	F	3	10	+ve
206	S	F	5	10	-ve
222	B	F	4	9.4	+ve
223	B	F	2	8.5	-ve

* x 10^7 cells

(F), female; (M), male; (S), Suffolk; (D), Dorset; (B), Blackface.

Table 8.3

Inoculation of lambs with tissue culture supernate and distrupted JS8 cell line passage 16.

Lamb	Breed	Sex	Age (hrs)	Inoculum	Tumour development
1597	D	M	12	50x	-ve
1598	D	F	12	50x	-ve
1596	D	F	6	100x	-ve
1557	D	F	6	100x	-ve
279	D	F	2	cell homogenate	-ve
288	D	F	2	ditto	-ve
289	D	F	2	ditto	+ve

50x and 100x represent the concentration of culture supernate.

(M), male; (F), female; (D), Dorset.

Only one of the 8 lambs inoculated with JS7 showed microscopic lesions of SPA (Table 8.4). Others showed neither macroscopic nor microscopic lesions. However, in 4 (80%) of the 5 lambs inoculated with JS8, microscopic lesions were detected (Table 8.5).

Pathology

The gross pathology, histology and ultrastructural characteristics of SPA lesions noted in lambs were similar in all experiments.

Gross pathology

Lesions typical of SPA were seen at necropsy in the lobes of affected lungs (Fig.8.1). The lesions appeared as diffuse or, in some areas of the lungs, small scattered greyish-pink consolidated tumour lesions of varying extent. Exudation of frothy fluid occurred from the trachea and cut surface of tumour lesions. Lesions were sometimes seen in all lobes, but were usually confined to the diaphragmatic lobes.

Microscopic lesions.

Histological examination of tumourous lungs revealed focal or widespread proliferation of alveolar lining cells. Alveoli were lined by continuous layers of cuboidal or columnar epithelial cells, giving an adenomatous appearance to the lung (Fig. 8.2). Some alveoli in which the proliferating epithelial cells completely occluded the lumena were scattered throughout the affected tissue (Fig. 8.3). Neoplastic changes were

Table 8.4

Oncogenicity of JS7 (passage 137) in neonatal lambs

Lamb number	Breed	Sex	Age (hrs)	Number of cells* inoculated	Tumour development
K710	B	M	24	10	-ve
K711	B	M	24	10	-ve
K712	B	M	24	10	+ve
K713	B	M	24	10	-ve
K714	B	M	24	5.1	-ve
K715	B	M	24	5.1	-ve
K716	B	F	12	5.1	-ve
K717	B	M	12	5.1	-ve

* $\times 10^7$ cells.

(M), male; (B), Blackface.

Table 8.5

Oncogenicity of JS8 (passage 125) in neonatal lambs.

Lamb number	Breed	Sex	Age (hrs)	Number of cells* inoculated	Tumour development
K1230	G	M	24	12	+ve
K1235	G	M	24	12	-ve
K1238	G	M	24	12	+ve
K1262	G	M	12	10	+ve
K1264	G	M	12	10	+ve

* x 10^7 cells.

(M), male; (G), Greyface.

Fig. 8.1

Macroscopic appearance of experimentally-induced SPA lung. Dorsal view showing nodular lesions of varying sizes in apical and cardiac lobes.

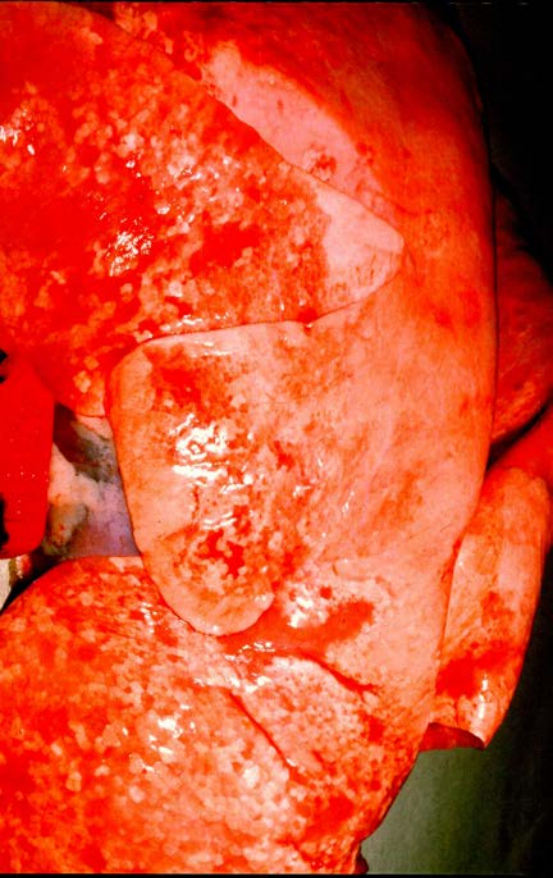
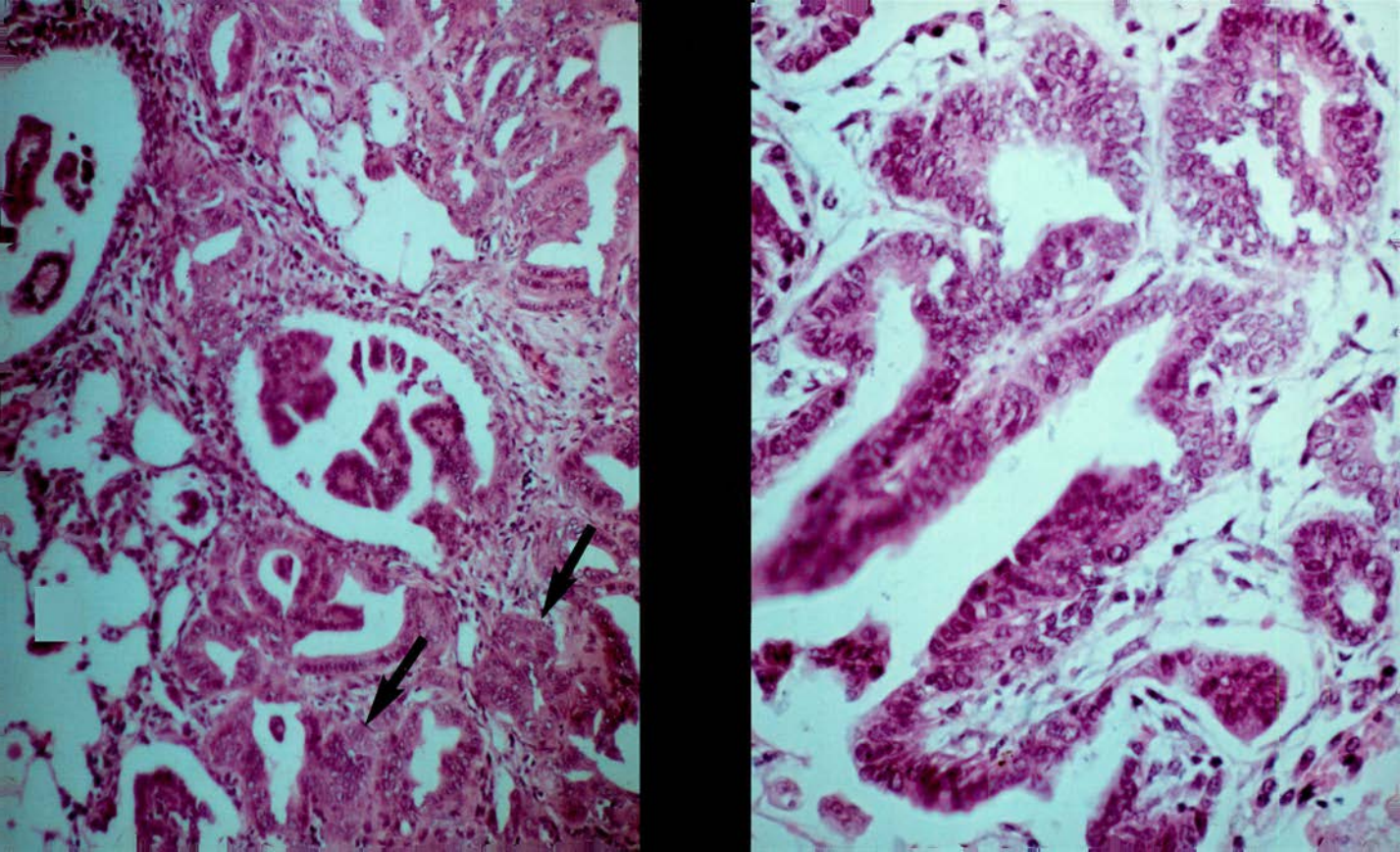


Fig. 8.2

Adenomatous alveolar epithelium supported by a thin stromal tissue. H & E stain, x 384

Fig. 8.3

Widespread SPA lesion in lamb lung. Note the epithelial proliferation of the bronchiolar and the alveoar cavities. Proliferation of epithelial cells has caused the blockage of some alveolai (arrows). H & E stain, x 102



also observed in the bronchioles (Fig. 8.4). Polyps of proliferating epithelial cells arising from several points in the alveolar or bronchiolar walls projected into their lumena. The cells comprising the polyps were regularly arranged in a single layer or several layers resting on narrow cores of loose connective tissue. In general, the alveoli seemed to be more severely affected than the bronchioles. Marked perivascular and peribronchiolar infiltration with lymphoid and plasma cells was seen, extending into the interstitial stroma. Exudates of serous fluid and pulmonary macrophages surrounding the primary foci were commonly seen.

Ultrastructural features of tumours

The fine structure of tumour lesions revealed cell shapes varying from cuboidal to columnar (Fig. 8.5). The tumour cells possessed microvilli on their surfaces and desmosomes between cells were evident. Nuclei in these cells tended to be heterogeneous in size, shape and electron density. They were round or oval with either smooth or indented membranes. Small peripheral rims of condensed chromatin and more than one prominent nucleolus were observed in each nucleus. The nucleoli were vesiculated and generally marginally located.

The cells contained round or oval electron-lucent cytosomes, usually situated apically, which occasionally exhibited lamellar internal structures (Fig. 8.6).

Fig. 8.4

Prominent intra-lumenal neoplastic growth from bronchiolar epithelium supported by a stalk in lung of lamb with SPA. H & E stain, x 102

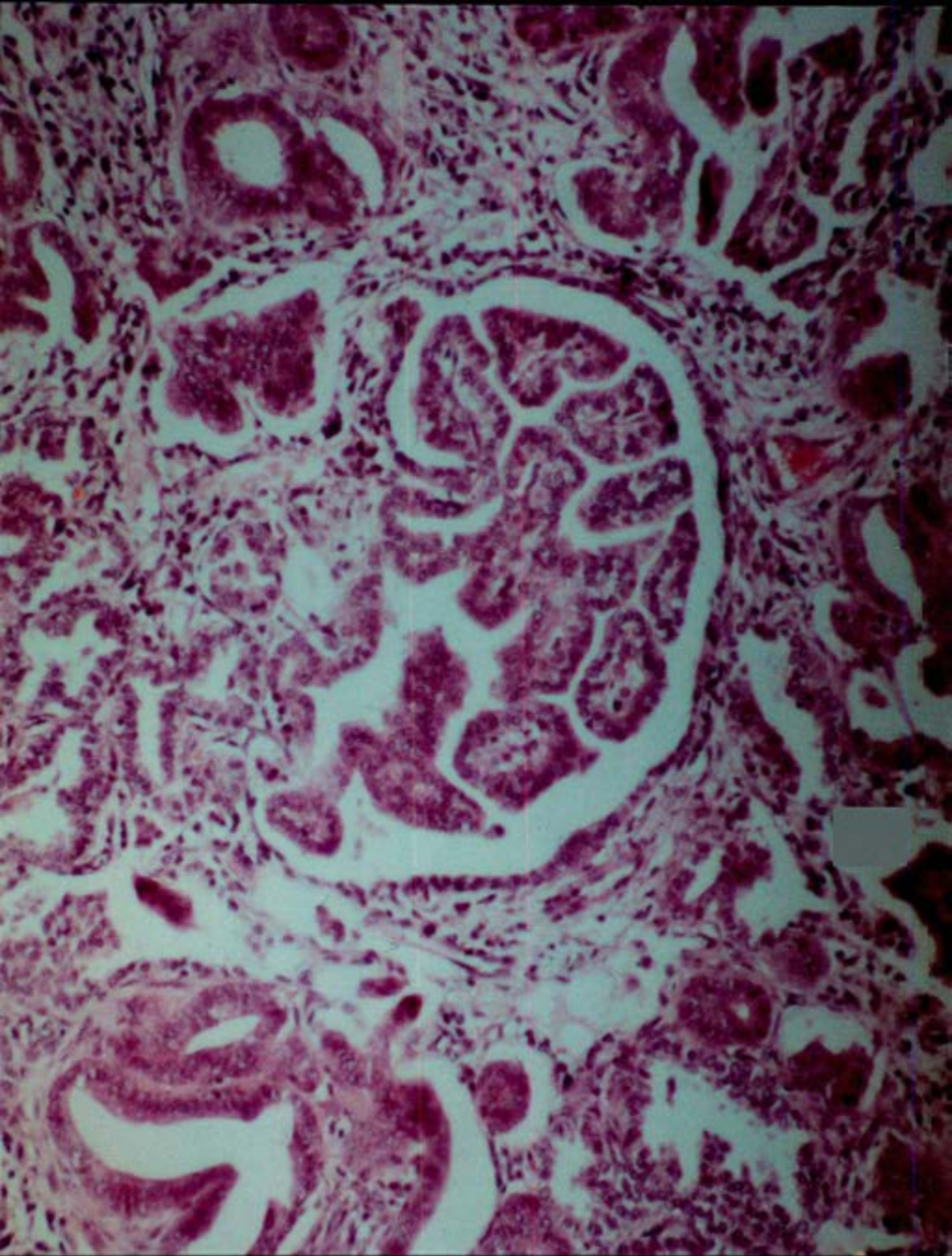


Fig. 8.5

Electron micrograph of lesion induced by JS8 cells (passage 16). The alveolar wall is lined by columnar cells with a uniform appearance. Note the presence of desmosomes, microvilli, and apical cytosomes. x 6000

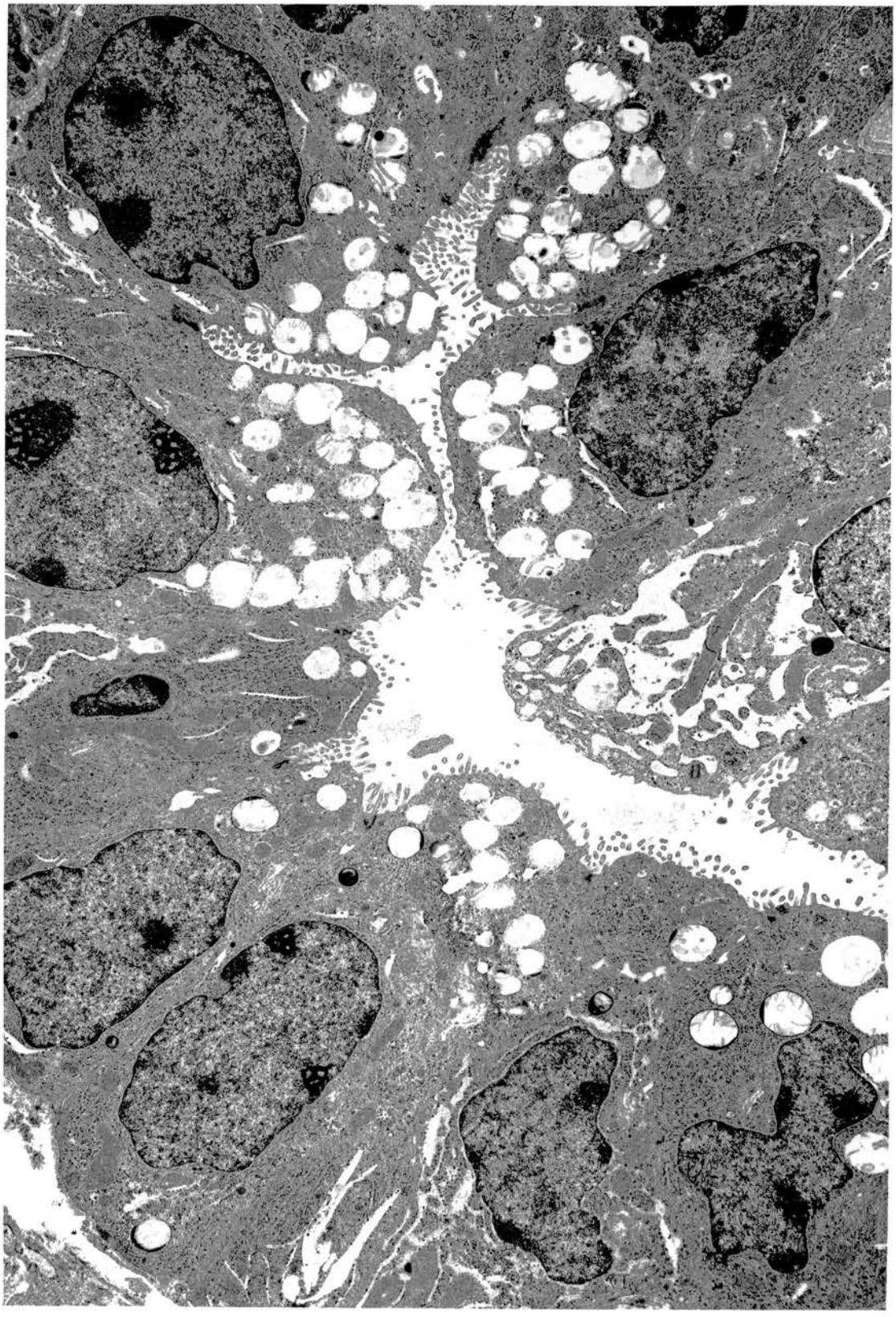
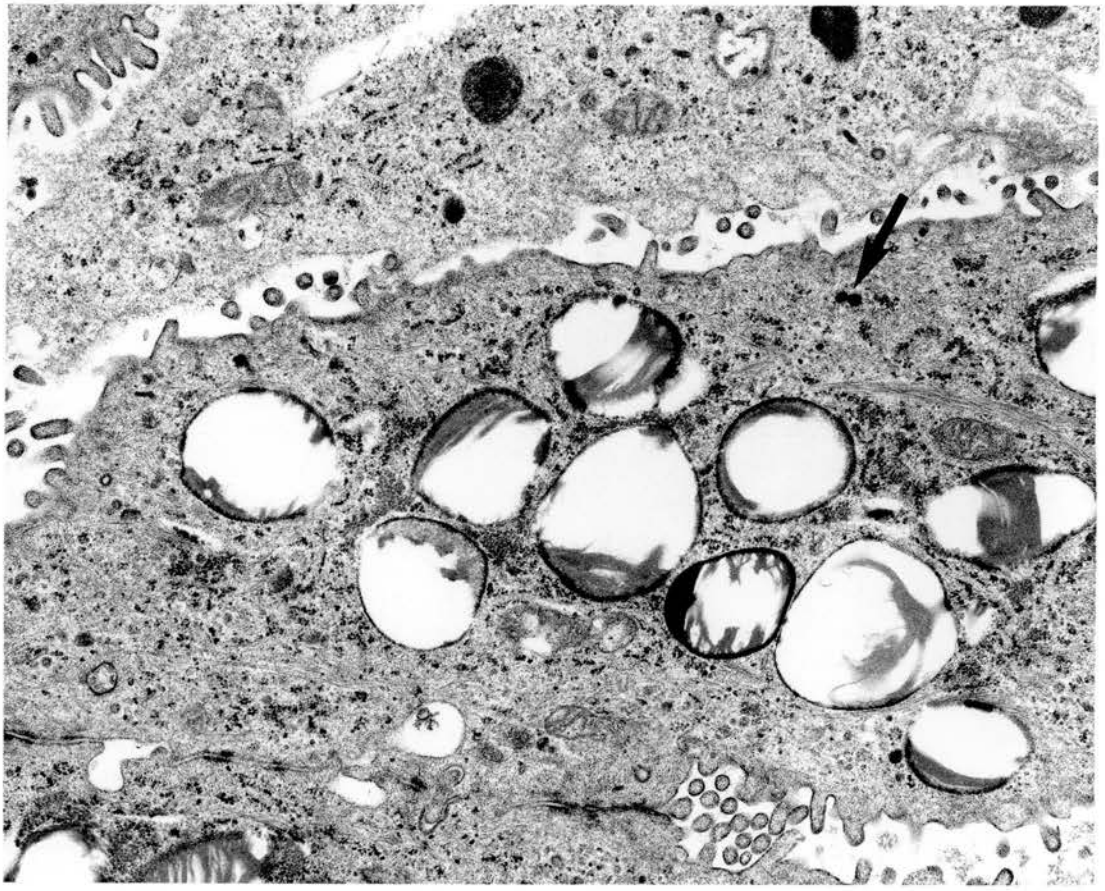


Fig. 8.6

Apical region of alveolar tumour cell showing lamellar bodies, desmosomes, microvilli and intracytoplasmic viral particles (arrow). x 15000.



Tumour cells contained moderate numbers of mitochondria, which appeared round or elongated. Although they usually showed an orthodox conformation with horizontal, regularly spaced cristae. In some instances mitochondria were moderately swollen and had lost some of their cristae. Endoplasmic reticulum was abundant in these cells and appeared as long meandering cisternae. Ribosomes were numerous and occurred freely or formed chains.

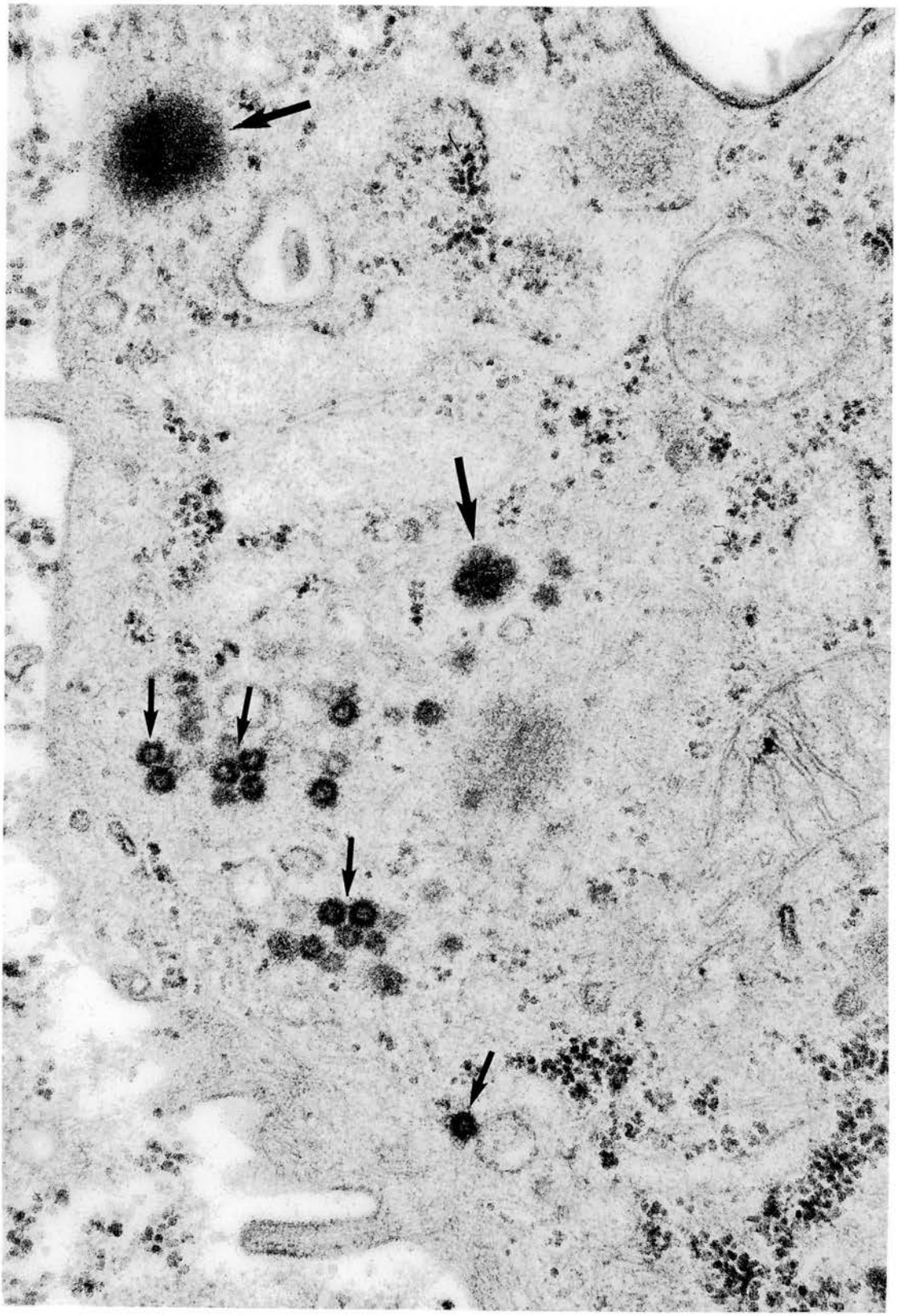
Cytoplasmic glycogen, a feature of developing type II pneumocytes (Kikkawa et al, 1968), was usually seen in small or large aggregates in most of the tumour cells.

Virus particles in tumour cells

Virus particles were found in lung tumour tissue from all 4 lambs examined (one lamb inoculated with JS8 and the other three with JS7). Spherical virus particles of 2 types were observed: namely, intracytoplasmic particles with electron lucent centres and extracellular particles with electron dense nucleoids. The intracytoplasmic particles resembled type A retrovirus. These particles contained three layers (Fig. 8.7). A dense ring surrounded a relatively electron-lucent central core; the outmost layer was of a moderate electron density. The diameter of the particles

Fig. 8.7

Apical region of an alveolar tumour cell. Note intracytolasmic A-type particles occurring singly or in groups (short arrows). Also cytoplasmic inclusions are evident (large arrows). Viral particles are clearly seen in the lower inclusion body. x 60000.



ranged from 72 nm to 80 nm and averaged 77 nm. They also were found budding from the cell microvilli (Fig. 8.8).

A-type virus particles were generally found in small quantities in tissue fragments obtained from SPA lungs. Single scattered particles, small groups of 2 to 6 particles, or sometimes larger groups of 10 to 25 particles could be found within one cell section.

Occasionally, areas of dense material with no obvious limiting membrane, and measuring up to 308 nm, were present in some cells (Fig. 8.7). Within these areas it was possible to distinguish electron dense ring shaped structures, measuring the same size as those of type A particles.

The extracellular particles (Fig. 8. 9) had outer membranes of the same thickness and density as the cellular plasma membrane, with occasional short knobs; internally they had dense, marginally located nucleoids. The size of the particles ranged from 80 nm to 115 nm in diameter. The particles were located in the extracellular spaces in the vicinity of the microvilli of the tumour cells. No C-type virus particles were found. Extracellular particles were present in fewer numbers than A type particles and always appeared singly.

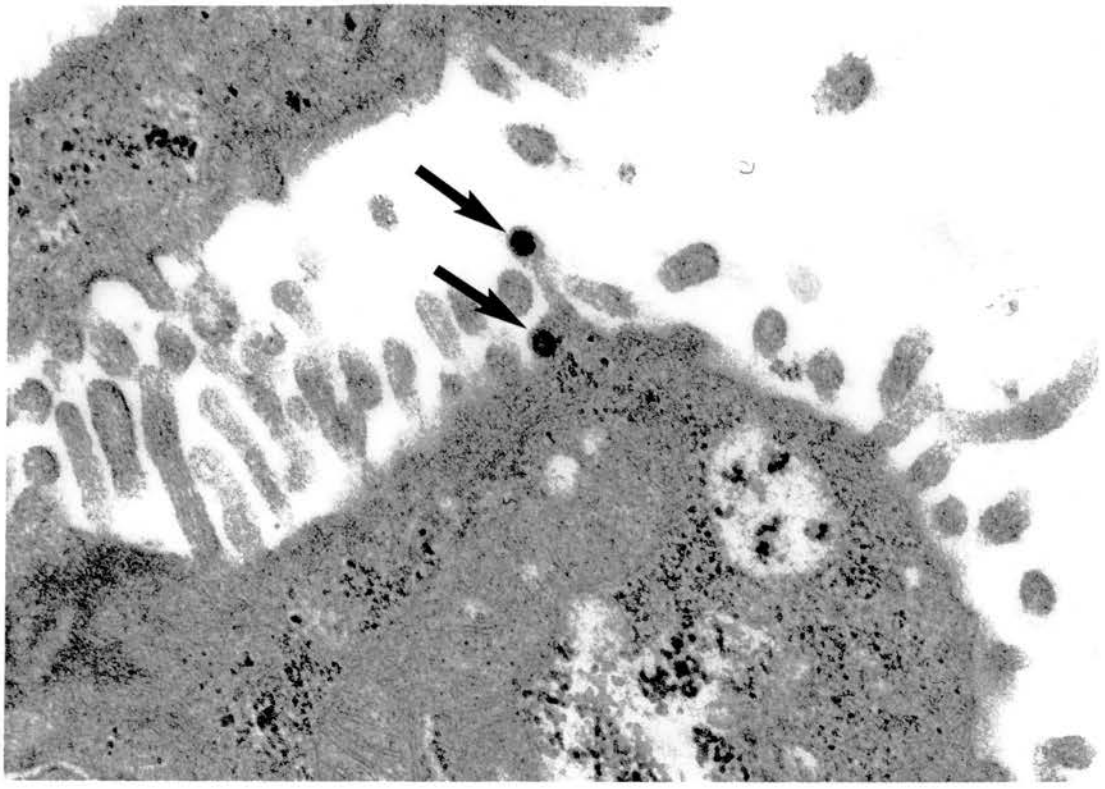
Cell cultures were initiated from the tumour tissue of four lambs. When supernates of cultures initiated from 4 lambs, were assayed by Western blotting, the SPARV P25 was detected (Fig.8.10). Similar findings were obtained with lung fluids from three lambs.

Fig. 8.8

Electron micrograph showing type A virus particles budding from the microvilli of the cell (arrows). x 4500.

Fig. 8.9

Electron micrograph showing extracellular virus particles (arrows). x 30000



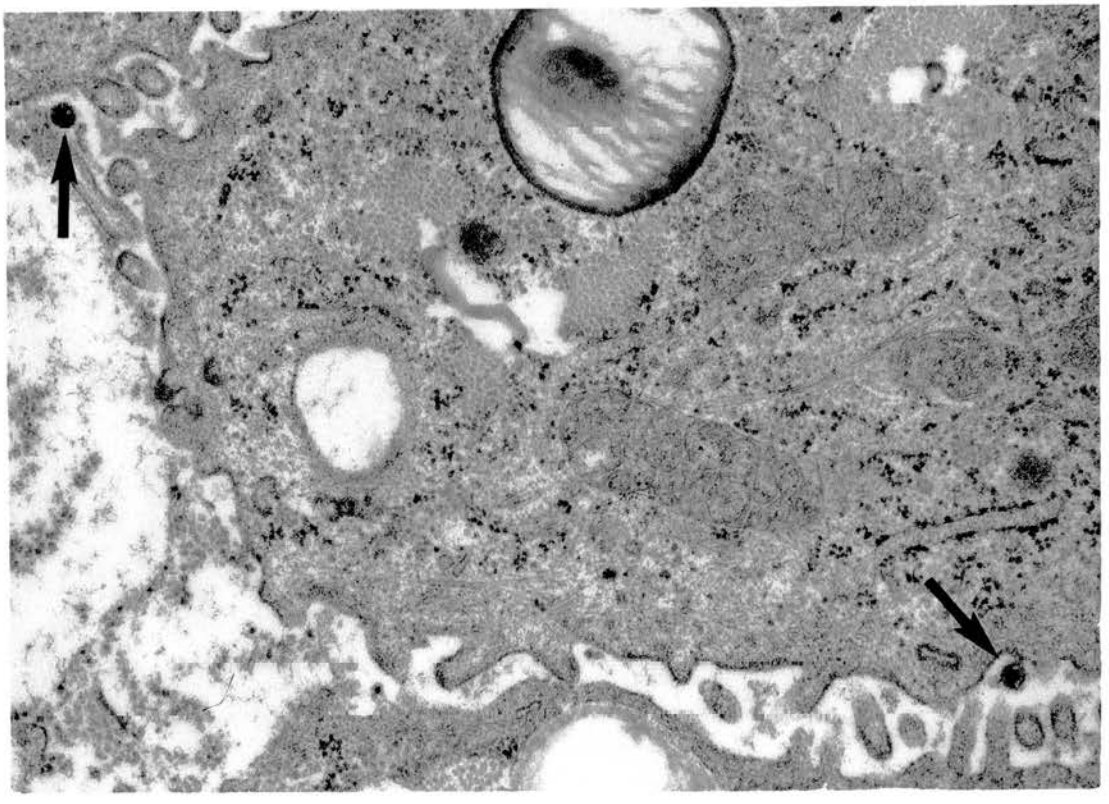


Fig. 8.10

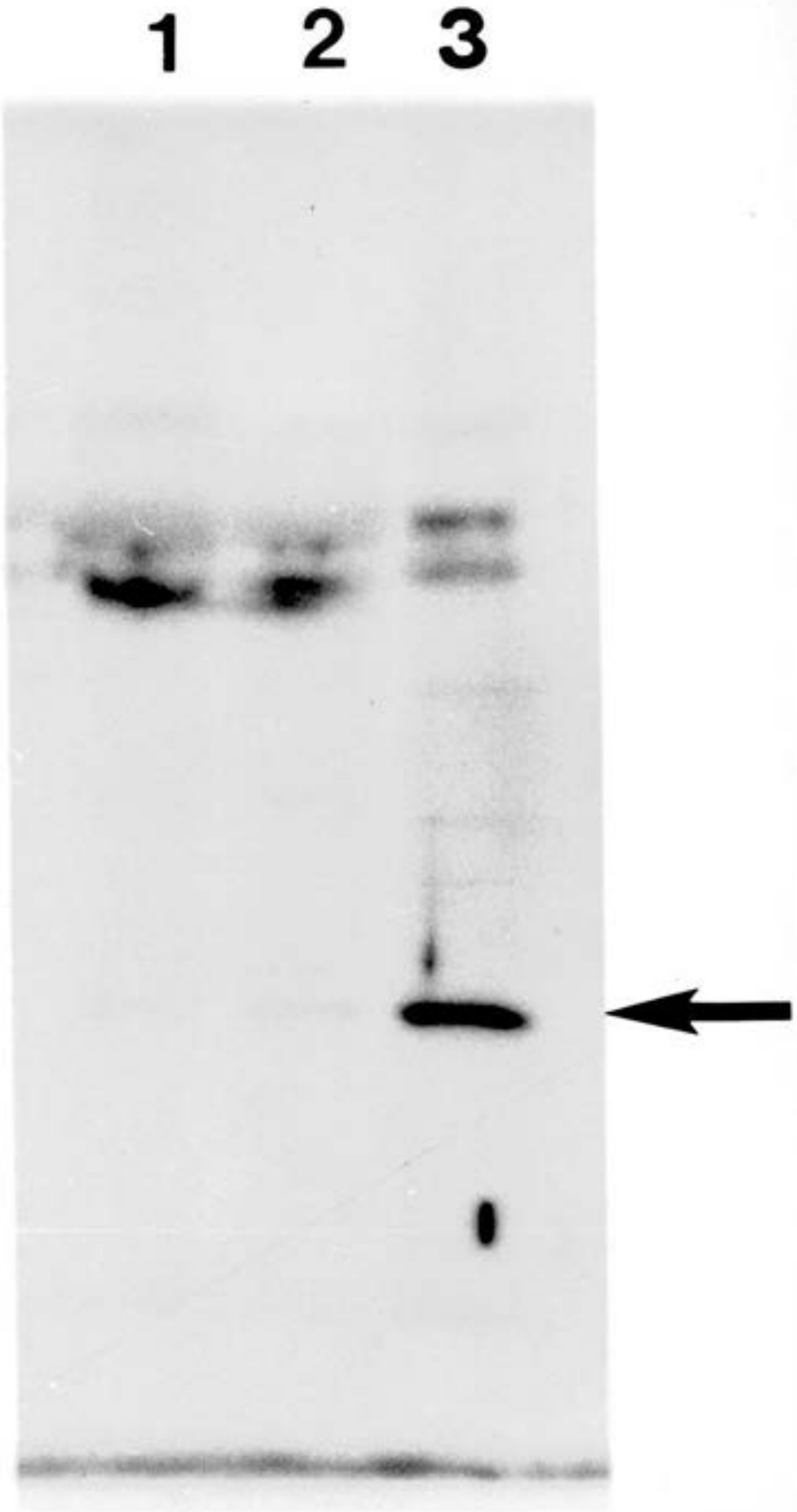
Immunoblot analysis of supernates derived from cultures initiated from experimentally induced SPA tumour in lambs inoculated with SPA cell lines.

Lane 3: supernate from primary culture, collected 24 hours after plating SPA tumour cells in culture.

Lane 2: supernate derived from passage 1 of SPA culture indicated in lane 3 and harvested after 24 hours' incubation.

Lane 1: supernate derived from passage 1 of SPA culture indicated in 2. Second harvest of the supernate collected between 24 and 48 hours' incubation.

Note a strong immunoreactive band corresponding to the SPA associated P25 in lane 3 (arrow), and faint bands in lane 1 and 2.


1
2
3

Chromosome analysis

Metaphase preparations of SPA cells were characterized karyologically at the first subculture in vitro.

The number of chromosomes in each cell culture was measured by counting 57 random metaphases. The modal number was 54 chromosomes, with 70-77% of the metaphases having 54±3 chromosomes. The other metaphases of the cells contained 45-50 or 104-108 chromosomes. The Y chromosome was detected in almost all the metaphases examined (Fig. 8.11).

Fig. 8.11

Metaphases in cells from cultures developed from experimentally induced SPA. Note the Y chromosome (arrows).

DISCUSSION

The induction of SPA in lambs not only confirms and extends other investigators' findings that SPA can be reproduced by cells grown *in vitro* (Coetzee *et al*, 1976), but also presents the first report that established SPA cell lines are capable of inducing the disease in lambs when inoculated at high passage levels (125 to 137 passages). Considering the known history of the Moredun breeding flocks and the age incidence of the natural disease, it is highly unlikely that any of the recorded lesions were attributed to spontaneous disease. Furthermore, the frequency of tumour induction produced by the cell lines at high passage levels was comparable to that obtained by the cells at low passages. These observations indicate that continuous *in vitro* propagation of these SPA cell lines does not result in loss of oncogenic potential.

Although the number of lambs used in the experiments reported in this study was small, several observations are of interest.

First, there were no demonstrable differences in the proportion of lambs developing SPA, whether induced by JS7 or JS8 cells. Secondly, the proportion of lambs developing SPA after inoculation of intact cells or cell homogenate were similar. Thirdly, the number of tumour cells inoculated is not the deciding factor for successful induction, as induction of SPA was achieved by inoculation of low cell numbers (3.3×10^7) as well as high numbers (12.5×10^7).

Although the number of male and female lambs inoculated with SPA cells and used in this study was not similar, the percentage of lambs that developed SPA lesions was higher in males (9/18; 50%) than in females (3/11; 27%), which is also in agreement with the findings of Verwoerd et al (1980a). These observations are not without precedent, because epidemiological evidence of the greater susceptibility of males has been reported (De kock, 1929; Mackay et al, 1971; DeMartini et al, 1985). The first worker found that 25% of castrated males were affected as against only 3% females. However, although Mackay et al (1971), based on experimental evidence, showed a significant increase in susceptibility of males to the disease, they indicated that their results were not conclusive. They further suggested that there may be a sex difference in susceptibility to SPA under certain conditions, such as influence of temperature. However, a survey in Peru, showed that male sheep, whether castrated or not, had a higher incidence of the disease (DeMartini et al, 1985). The increased susceptibility of male lambs may have been due to management or some other epidemiological factors rather than an inherent difference in susceptibility.

Homogenates of JS8 cells caused SPA lesions in one lamb. Thus it is evident that the cells do contain infective material. Similar results were demonstrated by Verwoerd et al (1980a). In contrast, cell-free supernates of culture fluid, of the same cell line and the same passage level from which the cell extracts were prepared, failed to produce SPA tumours. The results

suggest that low quantities of virus are released from these cells (Sharp et al, 1985); a view which is supported by previous experience which showed that lung fluid, in which the SPARV is readily demonstrated by reverse-transcriptase activity and Western blotting, induced clinical signs of SPA rapidly in almost 100% of inoculated lambs (Sharp et al, 1983; McCullough et al, 1985). In contrast, JS7 and JS8 SPA cell lines induced the tumour in only 41% of 31 inoculated lambs, and in only 6 of those did clinical signs appear. Further support for this view is that SPARV P25 was not detected in supernates of JS7 and JS8 cell lines at the time these experiments were conducted. Failure to induce tumour with cell-free supernate collected from primary culture of SPA has also been reported (Tustin and Geyer, 1971).

The gross and microscopic appearance of adenomatous tumour lesions in lung following intratracheal inoculation of cell homogenate, cultured cells or lung fluids has been amply documented in the literature, and it is perhaps sufficient to state that no difference was observed between the gross and microscopic appearance of lesions induced by the cells used in the present study and those induced by trypsinized cells or lung fluids reported by others (Tustin, 1969; Coetzee et al, 1976; Verwoerd et al, 1980a & 1980b; Sharp et al, 1983). Furthermore, the manifestations and lesions of the disease in the inoculated lambs were similar whether produced by high or low culture passages.

The results of the present experiments, as demonstrated by immunological and ultrastructural studies, indicate that SPA tumours are not caused by transplantation of the inoculated cells as suggested by Coetzee *et al* (1976) and Verwoerd *et al* (1980a), but rather result from transformation. The reasons for this conclusion are as follows. During *in vitro* passage over three years, no morphologic evidence of virus particles in the SPA cell lines JS7 and JS8 has been observed by electron microscopy or detected by immunoblotting, neither has virus replication been induced by various means. However, when tissues from the induced tumours were examined by electron microscopy, virus particles were found. Additionally, virus replication was indicated by the demonstration of SPA retrovirus specific antigen (P25) in lung fluid and in the supernate of cultures prepared from these tumours. The only source of SPARV in these lambs must have been the inoculated cells. Therefore, the compelling interpretation of these results is that SPARV, integrated within JS7 and JS8 genomes, has been reactivated and becomes available to transform other cells within the recipient lambs. Additional support for this hypothesis is the transmission of SPA to a lamb by a homogenate of JS8 cells. The mechanism by which the SPARV becomes reactivated is unclear at the present time but may involve *in vivo* derepression of cellular factors that restrict the full expression of the virus *in vitro* and/or

the action of some additional factors, such as hormones/ or other growth factors present in lamb lung.

Although it is difficult to completely exclude the occurrence of transplantation of the inoculated cells, several other observations indicate that it is unlikely or not a major event in tumour induction. First, the sex and karyotype of the induced tumour was always the same as the recipient (Fig. 8.11; Verwoerd et al, 1980a). Secondly, SPA cells inoculated subcutaneously or intravenously failed to induce tumours in lambs (Coetzee et al, 1976. J.M. Sharp, personal communication).

In this study, intracellular and mature extracellular virus particles with distinctive morphology were found in all 4 lambs examined. The intracellular particles were similar to type A retrovirus particles. Association of A virus particles with SPA tumour cells has been demonstrated previously in natural (Perk et al, 1971; Sharp et al, 1983) and also in experimentally induced SPA (Sharp et al, 1983; Angus et al, 1985; Payne et al, 1986). Type A particles are considered by several investigators as an intracellular precursor of type B and type D particles (Imai et al, 1966; Smith, 1967; Dalton and Potter, 1968). However, it is hard to establish if the virus particles observed in SPA tumour cells represent precursors of the extracellular particles and are the causal agent for SPA tumour.

Electron micrographs indicated that many of the SPA epithelial tumour cells contained intracytoplasmic type A particles but that in many cases, although they were located close to the plasma membrane, only a small proportion appeared to be actively budding into the extracellular space. This may indicate inhibition in the process of virus release from these cells. However, type A particles have been reported in a variety of neoplastic and normal mouse tissues (Wivel and Smith, 1971). Although the biological significance of type A particles is unclear, they have been reported to play a role in embyryogenesis and cellular differentiation as evidenced by their appearance in the early stages of mouse embryonic development and their disappearance as differentiation is achieved (Andersen and Jeppesen, 1972; Biczysko et al, 1973; Calarco and Szollosi, 1973).

The extracellular virus particles seen in SPA tumours closely resemble type B or type D particles in their morphology. These virions can be readily distinguished morphologically from type C particles in thin sections by the presence of long spikes on the surface of the virion, by the eccentric location of the nucleoid in mature extracellular particles, and by the presence of an intermediate layer between the core and viral envelope (Sarkar and Moore, 1970 & 1974; Sarkar et al, 1973; De Harven, 1974). Such virions have not only been demonstrated, but have been implicated as a causal agent of pulmonary adenomatosis on the basis of

morphological, antigenic, biochemical and transmission studies (Herring et al, 1983; Sharp et al, 1983, Sharp and Herring, 1983; Angus et al, 1985).

Electron-dense inclusion structures were observed in some tumour cells. Such structures were usually found in association with cells containing virus particles. In some of these structures viral particles were visible. Even more marked is the striking resemblance of the viral particles to type A particles observed in the cell cytoplasm. Therefore, based on these observations, the present study suggests that the inclusion structures may represent a site of virus assembly. Payne and Verwoerd (1984), however, had noticed an inclusion body with an electron-dense material in only one tumour cell, when 50 specimens were examined but, unfortunately, no further description was given.

CONCLUSIONS

1. The mechanism of SPA induction following inoculation of intact viable cells is a process of transformation rather than transplantation.
2. The oncogenic potential of the cell lines is maintained after numerous passes.
3. The retention of SPA producing potential provides strong support for the use of these cell lines as an *in vitro* model. The availability of transformed cells derived from the original tumour will be a valuable aid in future studies of SPA tumour.

DISCUSSION AND CONCLUSIONS

In the past, the problem of establishing permanent cell lines of SPA epithelial cells has received a lot of attention, but with little success (Coetzee et al, 1976). In most instances cultures have been maintained for only a few passages before the epithelial cells have either died or become overgrown by fibroblasts (J.M. Sharp, personal communication). There is general agreement that the complexity of lung tissue represents a major obstacle to the establishment of monolayer cultures enriched with transformed cells and that a means of overcoming these difficulties will be necessary before one can successfully establish SPA cell lines. This view was borne out by studies described in the present work. Although the technique developed in Chapter 3 produced more than 96% of type II pneumocytes, which represented all the viable cells in suspension, the overall viability of the cells in suspension was low. Therefore, further refinement of the procedure by removal of dead cells may be introduced to allow greater cell attachment, thereby improving growth of the tumour cells in culture. Removal of dead cells may be achieved by a variety of means such as gradient centrifugation through Percoll (Pertoft et al, 1977; Kurnick et al, 1979). Further unpublished studies in this laboratory have shown that dead cells can be removed from SPA cell suspension by Percoll density gradient centrifugation.

Thus, the development of a technique for the isolation of epithelial cells from SPA tumours with a high yield of type II alveolar cells opened the door for the establishment of no fewer than four cell lines from SPA tumours. The epithelial nature of the cells was evidenced by their cuboidal and closely apposed morphological appearance. They also retained many features of SPA epithelial cells taken directly from SPA tumours, namely the presence of microvilli and desmosomes (Chapter 4). In addition, the presence of lamellar bodies in the cytoplasm of the SPA cells was the main criterion used to determine their origin and identity.

However, the problem of the cellular origin of the SPA cell lines is complicated by two circumstances: (a) the lack of secure morphological or biochemical markers for undifferentiated type II cells i.e. cells which lack lamellar bodies; and (b) the cellular heterogeneity of the lung. Therefore, the SPA cell lines were assessed in a number of ways both <u>in vitro</u> and <u>in vivo</u> (Chapter 4,5,6) and it was found that the cells demonstrated a striking stability of epithelial features in all tests. These features were detected in cells in monolayers, soft agar, and nude mice tumours. In addition the cells maintained a "memory" for the same spatial relationships observed in the original tumours which is indicated by the papillary growth in soft agar and nude mice (Chapter 4,7).

JS7 cells were found to possess the lamellar structures similar to those seen in normal type II pneumocytes *in vivo*, and in this respect they most closely resembled type II pneumocytes. In contrast, JS8 cells lacked the characteristic lamellar bodies and for this reason it was difficult to judge the identity of this cell line. However, the results presented in Chapter 5 showed that bromhexine hydrochloride induced lamellar body formation in JS8 cells as well as increases in size and number of these structures in JS7 cells. These observations indicate that both JS7 and JS8 cells probably originate from type II cells. Furthermore, because JS7 and JS8 showed different features with regard to the stage of differentiation, it implies that different states of differentiation are possible and that these can be stable. Although the observations during these studies indicate that these two cell lines are probably derived from transformed type II pneumocytes, more definitive information regarding their lineage should be sought from functional studies e.g. labelling with choline chloride and analysis of surfactant production.

In the context of the relationship of the SPARV to the SPA cells it is of interest that no virus particles similar to SPA retrovirus were detected in tumours in nude mice, and that epithelial tumour cells from nude mice tumour did not produce virus when grown in

culture (Chapter 7). This matches well with the failure to find the virus in the parent cells at the time of inoculation. The lack of SPARV production in vitro by SPA cells derived from immunologically defective nude mice seems to suggest that the continued repression of this function is not dependent on host responses but rather on some intrinsic cellular mechanism or virus defectiveness.

In addition, and perhaps more important, the transmission experiments provided the first evidence that SPA cell lines remained infective despite the high passage levels in vitro. When these cells were inoculated intratracheally into neonatal lambs typical SPA was produced in which the histological and ultrastructural features were indistinguishable from the SPA tumour induced by inoculation of tumour homogenates or lung fluids (Verwoerd et al, 1980a & 1980b; Sharp et al, 1983; Angus et al, 1985; McCullough et al, 1985; Rosadio, 1987). Virus particles were detected in the target cells and cultures initiated from these tumours contained virus, as measured by Western blotting. However, the failure to reactivate the virus following inoculation of cells in nude mice compared to its activation in lambs suggests that specific factor(s) must be present in lambs which mediated virus expression. Hitherto, experimental transmission studies relied on lung tumour homogenates or lung fluids. Such materials are not easy to obtain. Therefore, JS7 and JS8 cell lines should be seriously

considered as an important alternative source of material for the production of SPA.

The establishment of these cell lines from SPA tumours has fulfilled a major requirement that many investigators considered essential. It has not been proven that SPARV is the transforming agent in these cells, but the detection of virus antigen at early passes of these cell lines, the development of typical SPA by inoculation of lambs with these cell lines, the presence of virus in lung tumour in situ and its antigen in culture, would tend to implicate SPARV as the transforming agent. The best evidence to confirm the role of SPARV as the agent of SPA would be direct in vitro transformation of normal type II alveolar cells by this virus.

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APPENDICES

APPENDIX 1

Media and buffers

Ham's F12K Medium

Inorganic salts

	mg/litre
$CaCl_2$ (anhydrous)	135.00
$CuSO_4.5H_2O$	0.002
$FeSO_4.7H_2O$	0.80
KCl	285.00
KH_2PO_4	59.00
$MgCl_2.6H_2O$	106.00
$MgSO_4.7H_2O$	393.00
NaCl	7530.00
$NaHCO_3$	-
$Na_2HPO_4.7H_2O$	218.00
$ZnSO_4.7H_2O$	0.144

Other components

	mg/litre
Glucose	1260.0
Hypoxanthine	4.0
Lipoic acid	0.206
Phenol red	3.0
Putrescine.2HCl	0.322
Sodium pyruvate	220.0
Thymidine	0.700

Amino Acids	mg/litre
L-Alanine	17.0
L-Arginine HCl	422.0
L-Asparagine H_2O	30.0
L-Aspartic Acid	26.6
L-Cysteine HCl.H_2O	70.04
L-Glutamic Acid	29.4
L-Glutamine	292.0
Glycine	15.0
L-Histidine HCl.H_2O	41.9
L-Isoleucine	7.88
L-Leucine	26.20
L-Lysine HCl	73.0
L-Methionine	8.96
L-Phenylalanine	9.92
L-Proline	69.06
L-Serine	21.0
L-Threonine	23.8
L-Tryptophan	4.08
L-Tyrosine	10.88
L-Valine	23.4

Vitamins	mg/litre
d-Biotin	0.07
D-Ca Pantothenate	0.477
Choline Chloride	13.8
Folic Acid	1.32
i-Inositol	18.00

Vitamins contin'd.	mg/litre
Nicotinamide	0.037
Pyridoxine HCl	0.062
Riboflavin	0.04
Thiamine HCl	0.337
Vitamin B_{12}	1.36

MED D-Val

Inorganic Salts	mg/ml
Calcium chloride ($CaCl_2$)	200.0
Potassium chloride (KCl)	400.0
Magnesium sulphate ($MgSO_3.7H_2O$)	200.0
Sodium chloride (NaCl)	6800.0
Sodium hydrogen carbonate ($NaHCO_3$)	2200.0
Sodium di-hydrogen phosphate ($NaH_2PO_4.H_2O$	140.0
Other Components	
Glucose	1000.0
Phenol red	10.0
Amino Acids	
L-Arginine HCl	126.00
L-Cystine	24.00
L-Glutamine	292.00
L-Histidine HCl. H_2O	41.92
L-Isoleucine	52.46
L-Leucine	52.46
L-Lysine HCl	73.06

Amino acids contin'd.	mg/litre
L-Methionine	14.92
L-Phenylalanine	33.00
L-Threonine	47.64
L-Tryptophan	10.20
L-Tyrosine	36.22
D-Valine	92.00

Vitamins	
D-Ca pantothenate	1.00
Choline CI	1.00
Folic acid	1.00
L-Inositol	2.00
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

Phosphate buffer saline (PBS)

	mg/litre
Sodium chloride (Na Cl)	8000
Potassium chloride (K Cl)	200
di-sodium hydrogen orthophosphate (Na_2HPO_4)	1150
Potassium di-hydrogen orthophosphate (KH_2PO_4)	200

Hanks' Balanced Salt Solution (HBSS)

	mg/litre
Magnesium sulphate ($MgSO_4 . H_2O$)	200
Magnesium chloride ($MgCl_2 . 6H_2O$)	100
Calcium chloride ($CaCl_2 . 2H_2O$)	140
Sodium chloride (NaCl)	8000
Potassium chloride (KCl)	400
di-sodium hydrogen orthophosphate ($Na_2HPO_4 . 7H_2O$)	3000
Potassium di-hydrogen phosphate (KH_2PO_4)	60

Appendix 2

Mean volumes and standard errors of tumour growth in nude mice induced by JS7, JS8, JS14, JS15, A549 and NBL12 cell lines measured at 3 day intervals

Means

Measurements (days)	cell lines JS7	JS8	JS14	JS15	A549	NBL12
5	147±47	122±55	145±27.7	247±27.9	-	-
8	349±133	199±63	364±65	787±282	132±57	-
11	400±130	260±87	375±65	978±337	140±62	-
14	771±254	307±74	355±84	1169±284	145±61	-
17	839±281	330±68	331±94	1302±327	166±92	86.5±14.2
20	853±279	339±62	250±49.2	1347±300	189±85	96.2±19.3
23	848±282	339±62	146.4±37.6	1408±291	249±130	135±33.1
26	853±279	339±62	71.5±42.7	1431±273	383±211	147±46
29	851±282	339±62	52.8±24.1	1457±270	495±229	229±71
32	844±274	344±61	48±19.3	1462±182	603±244	353±121
35	856±267	350±60	41.4±16	1469±181	736±267	437±193
38	848±282	344±61	35.5±13.3	1476±298	885±287	556±206
41	850±281	344±61	30±10.5	1463±301	1030±273	709±282

Appendix 3

Chromosome composition of JS7(M) and JS8(M) cells

Chromosome composition of JS7(M) cells

Spread	Chrom.no.	Metacent.	Submeta.	Acro.	Telo.	Dicen.
1	32	2	4	1	25	
2	34	7	3	6	18	
3	38	7	3	2	26	
4	38	8	2	3	25	
5	39	6	2	3	26	2
6	40	11	1	9	19	
7	46	6	5	6	29	
8	48	7	5	4	32	
9	56	10	4	3	39	
10	56	10	3	-	41	2
11	60	10	6	5	39	
12	60	14	5	4	37	
13	65	12	6	2	45	
14	65	14	4	5	42	
15	68	14	2	4	48	
16	68	16	5	13	34	
17	69	9	5	2	42	1
18	69	13	3	2	51	
19	69	15	4	4	46	
20	71	18	6	10	39	
21	72	14	2	3	51	
22	76	19	5	7	45	
23	77	16	4	8	49	
24	78	13	6	7	51	1

Chromosome composition of JS7(M) cells contin'd.

25	84	19	3	4	58	
26	86	18	5	12	51	
27	100	22	4	5	69	

Chromosome composition of JS8(M) cells

1	53	6	4	8	35	
2	55	11	5	5	34	
3	56	6	3	2	43	2
4	59	8	4	5	42	
5	60	8	5	8	38	1
6	68	11	5	2	50	
7	68	14	5	3	46	
8	68	15	5	2	46	
9	71	6	5	8	52	
10	71	8	6	3	54	
11	72	11	8	2	51	
12	73	7	7	4	55	
13	73	13	6	3	51	
14	74	12	3	3	56	
15	74	14	5	2	53	
16	74	10	5	6	53	
17	74	12	5	8	49	
18	74	11	6	3	54	
19	74	10	4	4	56	
20	74	12	2	4	56	
21	74	5	7	5	57	

Chromosome composition of JS8(M) contin'd.

22	75	12	7	5	51	
23	75	7	3	4	61	
24	75	10	3	4	58	
25	75	9	5	5	56	
26	75	14	3	4	54	
27	76	16	3	2	55	
28	76	11	7	3	54	
29	77	13	3	3	58	
30	77	14	4	8	51	
31	77	9	7	2	59	
32	77	9	6	4	58	
33	77	11	3	4	59	
34	77	13	6	5	53	
35	78	9	8	5	56	
36	78	13	5	5	55	
37	80	9	5	5	61	
38	83	14	7	4	58	
39	89	11	6	3	69	3
40	91	16	6	5	70	
40	96	6	1	7	79	

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BRITISH ASSOCIATION FOR CANCER RESEARCH

EUROPEAN ASSOCIATION FOR CANCER RESEARCH

AND

ROYAL SOCIETY OF MEDICINE
(ONCOLOGY SECTION)

Joint Winter Meeting

The Royal Society of Medicine

1 Wimpole Street

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November 3rd-5th 1986

O.1

GROWTH AND MORPHOLOGY OF A CELL LINE FROM JAAGSIEKTE, A CONTAGIOUS LUNG TUMOUR OF SHEEP, CAN BE MAINPULATED IN VITRO.

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Jaagsiekte is a contagious lung tumour of sheep in which two secretory epithelial cells in the lower respiratory tract are transformed. These cells are type II pneumocytes in the alveoli and the cells of Clara in the terminal bronchioles.

A cell line (JS7) has been established from the lungs of sheep with jaagsiekte and has been propagated continuously in vitro for more than 140 passages. The cells possess properties of transformed cells such as growth in soft agar and athymic mice, yet retain many of the characteristic differentiated features of type II pneuocytes.

The effect of bromohexine HCl and prednisolone on the morphology and ultrastructure of JS7 cells was studied. Cells were cultured for up to six days in medium with various concentrations of the two chemicals.

Cells treated with prednisolone lost their squamous epithelial shape and assumed a fusiform swirling appearance. Coincident with this change in morphology, they also lost the characteristic cytoplasmic lamellar bodies but not apical microvilli nor desmosomes.

Cells treated with bromohexine HCl showed an increase in the number and size of lamellar bodies.

These data indicate that differentiated features of this important pulmonary epithelial cell can be manipulated in vitro.

O.2

Chemosensitivity Testing of Human Lung Cancer Cell Lines using a Colorimetric Assay, J. Carmichael*[1,2], W.B. De Graff[2], J. Gamson[2], A.F. Gazdar[1], J.D. Minna[1], and J.B. Mitchell[2], [1]Naval Med. Oncol. Branch, NCI and Naval Hospital, Bethesda, Maryland, U.S.A.

A colorimetric assay was used to determine the chemosensitivity profile of 30 human lung cancer cell lines. The assay is based on the cellular reduction of MTT, a tetrazolium salt, to a purple coloured formazan product which can be measured spectrophotometrically, with production of formazan proportional to viable cell number. Of 15 small cell lung cancer lines (SCLC) tested, 7 were derived from previously untreated patients (Un/T). The non-small cell lung cancer lines (NSCLC) comprised 4 adenocarcinoma, 3 large cell, 2 adenosquamous, 2 bronchioloalveolar, 2 squamous and 2 mesothelioma cell lines. Chemosensitivity was assessed following continuous exposure to drugs using a 4 day assay, with the ID_{50} defined as the dose of drug resulting in a 50% reduction in MTT formazan production. Representative ID_{50} values are illustrated in the following table:

	Un/T SCLC (n=7)	T-SCLC (n=8)	NSCLC (n=15)
Melphalan (μM)	3.98	17.28	37.04
Cis-Platinum (μM)	0.88	2.54	4.03
Adriamycin (nM)	26.28	167.24	205.40
VP-16 (μM)	1.24	10.26	31.44

For all 4 drugs, Un/T SCLC lines were more sensitive than T-SCLC lines, with the NSCLC lines least sensitive. These findings are in keeping with clinical experience using chemotherapy in these cell types. This colorimetric assay can be semi-automated and therefore offers a rapid, reproducible assay for the chemosensitivity testing of cell lines.

Research in Veterinary Science 1987, 43, 407–409

Three-step procedure for isolation of epithelial cells from the lungs of sheep with jaagsiekte

F. A. JASSIM, J. M. SHARP, P. D. MARINELLO, *Moredun Research Institute, 408 Gilmerton Road, Edinburgh*

An efficient and reproducible technique is described for the isolation of transformed sheep pulmonary adenomatosis cells. It includes three basic steps: prolonged trypsinisation to kill fibroblasts, magnetic removal of macrophages and adherence to remove the rapidly adherent cells. The resultant preparations of lung cells were enriched to 96·6 per cent type 2 pneumocytes.

PULMONARY adenomatosis (SPA, jaagsiekte) is a contagious lung tumour of sheep in which type 2 pneumocytes in the alveoli and cells of Clara in the terminal bronchioles are transformed (Nisbet et al 1971).

Cell cultures have been initiated previously from SPA tumours (Coetzee et al 1976) but these have not resulted in permanent cell lines. Furthermore, little information is available on the reproducibility of this procedure or the yield of cells with characteristics of tumour cells. There is, therefore, a need to develop techniques for the isolation of SPA cells for further studies and to develop permanent cell lines which retain the characteristics of SPA tumour cells.

The successful establishment of epithelial tumour cells requires that two major problems be surmounted: the overgrowth of epithelial cells by fibroblasts and the elimination of tumour macrophages, which if not removed can be activated in vitro and kill the tumour cells (Evans 1972). The proliferation of fibroblasts may be controlled either by their removal during the dissociation and isolation procedure or by inhibition of their growth in vitro (Williams et al 1971, Owens et al 1974, Gilbert and Migeon 1975, Coetzee et al 1976, Kao and Prockop 1977).

Several of these approaches were attempted in the authors' preliminary studies but proved insufficient to prevent the proliferation of fibroblasts or to sustain growth of epithelial cells. In the present study, a combination of three procedures was used successfully to obtain almost pure preparations of SPA tumour cells.

Prolonged trypsinisation has been reported to kill fibroblasts without any deleterious effects on epithelial cells (Coetzee et al 1976). Pieces selected from SPA tumours were cut into small fragments, and dissociated by gently stirring at 4°C for 48 hours in 0·25 per cent trypsin solution supplemented with 1 per cent chicken serum to protect cells (Douglas and Kaighn 1974).

Following trypsin dissociation, phagocytic cells were removed by the carbonyl-iron technique (Buick and Salmon 1980). Before use, the carbonyl-iron powder was washed three times in phosphate buffered saline (pH 7·2) and once in medium to remove toxicity. A suspension of 10^8 cells in 20 ml medium containing 1 per cent fetal bovine serum and 200 mg carbonyl-iron was incubated while slowly rotating for one hour at 37°C. Cells that had ingested carbonyl-iron particles and free carbonyl-iron particles were depleted from the suspension by attraction to a magnet and the remaining non-phagocytic cells were collected. This procedure was repeated until it was considered by visual inspection that all the carbonyl-iron had been removed.

The third and final step in the isolation procedure was differential adherence (Mason et al 1976) which was employed to separate epithelial cells from the remaining more rapidly adherent fibroblasts. The non-phagocytic cells were washed twice in growth medium and then plated in plastic flasks at 10^5 cells cm^{-2} and incubated for three hours at 37°C in a gas mixture (5 per cent carbon dioxide, 5 per cent oxygen, 90 per cent nitrogen). The non-adherent cells were aspirated and re-seeded in plastic flasks.

At every step, total cell count and viability were determined by trypan blue, and the proportion of type 2 pneumocytes determined by staining with Phosphine 3R (Pfleger 1977).

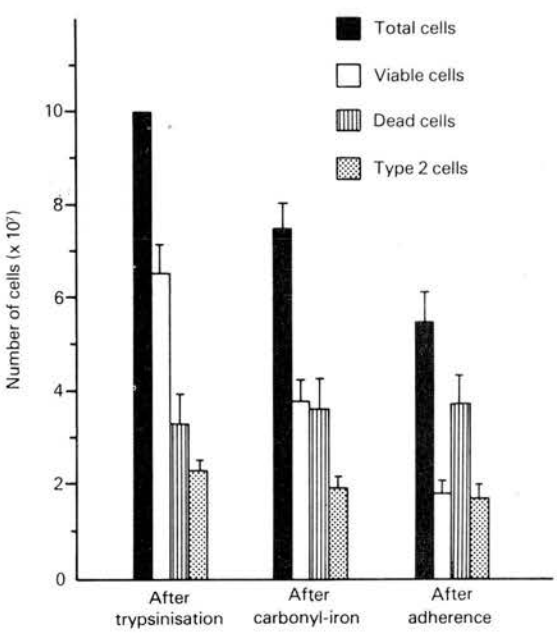

FIG 1: Effect of the isolation procedure on the viability and yield of type 2 pneumocytes from trypsin-dissociated sheep pulmonary adenomatosis tumours

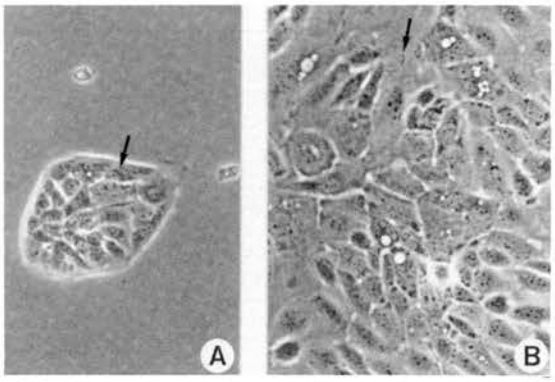

FIG 2: Primary cultures of sheep pulmonary adenomatosis cells. (A) 36 hours after plating; (B) four days after plating. The cells show features of type 2 pneumocytes, such as epithelial morphology and intracytoplasmic granules by phase contrast microscopy (arrows). × 130

Cells were grown in Ham's F12K medium (Kaighn 1973) to which 10 per cent fetal bovine serum had been added. The medium was further supplemented with 10 μg ml^{-1} bovine insulin during the first few early passages of cultures.

Results of these experiments are presented in Fig 1 and were obtained from six different tumours. In a population of 10^8 cells of unfractionated SPA cell suspension, the average yield of viable cells after trypsinisation was $67{\cdot}2 \pm 6{\cdot}4 \times 10^6$, of which $20{\cdot}3 \times 10^6$ (30·2 per cent) cells stained positively with Phosphine 3R.

Following carbonyl-iron treatment of the cell suspension, an average total of $27{\cdot}4 \times 10^6$ (40·8 per cent) cells was removed, leaving $39{\cdot}8 \times 10^6$ viable cells. Of these remaining viable cells the average number staining with Phosphine 3R was $19{\cdot}3 \times 10^6$ representing 48·5 per cent.

The adherence procedure further reduced the viable cells by $22{\cdot}1 \times 10^6$ indicating that 55·5 per cent were rapidly adherent cells such as fibroblasts and macrophages. The remaining $17{\cdot}7 \times 10^6$ viable cells contained $17{\cdot}1 \times 10^6$ (96·6 per cent) cells staining with Phosphine 3R which represents a loss during the three steps of only $3{\cdot}2 \times 10^6$ (15·8 per cent) of this cell type.

In culture the cells tended to form islands, from which they grew out progressively to form monolayers (Fig 2). Cells of the islands and confluent monolayers were cuboidal, closely packed and contained vacuoles and intracytoplasmic granules.

Identification of type 2 cells is based initially on their histochemical, morphological and ultrastructural properties. In this study, the type 2 cells were identified by their characteristic fluorescence with Phosphine 3R and the detection of refractile granules in the cytoplasm, usually in the perinuclear region. These criteria appear to be reliable markers of the transformed type 2 cells in SPA tumour because in other experiments, macrophages and both fibroblasts and epithelial cells obtained from normal lung have not demonstrated these features (authors' unpublished observations). Electron microscopy could not be used to confirm the identity because the residual iron particles in the cell suspension presented a technical difficulty to cutting ultrathin sections. A similar problem was encountered by Lafranconi et al (1983).

The method reported in the present study was efficient, reproducible and eliminated most of the fibroblasts and macrophages from the mixed cell suspension before placing in culture. The method yielded cell suspensions enriched to 96·6 per cent purity for epithelial type 2 cells from SPA tumour. These results are comparable to those obtained from rabbit lung by Lafranconi et al (1983) with a combination of three basic techniques; critical enzyme placement, magnetic removal of macrophages, and cell sizing through sieves to remove contaminating cell types.

During the three step procedure both the total number and proportion of viable cells were reduced markedly (Fig 1), whereas the number of dead cells remained constant. This indicates that the treatment with carbonyl-iron and adherence was removing only viable cells and that the reduction in viable cells can be attributed to the removal of phagocytic cells and fibroblasts. In contrast, the total number of type 2 cells was reduced by only $3{\cdot}2 \times 10^6$ and their proportion increased from 30·2 per cent to 96·6 per cent.

Pfleger (1977) has reported that Phosphine 3R produces the characteristic fluorescence and pattern of granules only in viable type 2 cells. Therefore, in the present experiment the increasing proportion of type 2 cells appears to be due to an enrichment of viable type 2 cells rather than to an accumulation of dead cells, which also would not be removed by the procedures employed.

Using the reported procedure the authors were able to isolate and propagate healthy epithelial cultures from all cases of SPA. Two of the cultures isolated by this method have now been established as permanent cell lines, and have been passaged more than 140 times in vitro.

Although replication of the SPA retrovirus was demonstrated in five of the six cell cultures by Western blotting during early passes, numerous attempts to induce replication in these two permanent cell lines have been unsuccessful (authors' unpublished observations). Similarly, contamination of the two cell lines by *Mycoplasma* species has not been demonstrated (A. Rae, personal communication).

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Experimental transmission of sheep pulmonary adenomatosis to a goat

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Veterinary Record (1986) **119**, 245

SHEEP pulmonary adenomatosis or jaagsiekte is a contagious lung tumour of sheep which occurs in many countries (Sharp and Martin 1983). Although a similar disease has been reported in goats, the published evidence does not present a clear picture of the morphological and histological features (Cuba-Caparo and others 1961, Rajya and Singh 1964, Tiwari and Pandit 1967). In view of the increasing numbers of goats in the United Kingdom and the high prevalence of pulmonary adenomatosis in sheep in Scotland (Hunter and Munro 1983, Ross and Williams 1983) a small experiment was conducted to determine whether a disease similar to sheep pulmonary adenomatosis could be produced in goats. The resulting lesions were compared with those which occur in sheep.

Rapid transmission of sheep pulmonary adenomatosis can be achieved reliably by intratracheal inoculation of newborn lambs with lung fluids obtained from sheep clinically affected with the disease (Sharp and others 1983). This procedure was therefore used to attempt to transmit the disease to newborn goats. Three Anglo-Nubian goat kids, less than 24 hours old, were inoculated intratracheally with 5 ml of lung fluid from infected sheep; it had been concentrated 15 times and contained the retrovirus which causes the disease (Sharp and others 1983). Two lambs of similar age were inoculated with the same material and served as controls for infectivity.

All the animals were reared with their dams in isolated accommodation. Within three weeks both lambs had clinical evidence of pulmonary adenomatosis and by six weeks they had developed widespread tumour lesions in the lungs, which were confirmed by histological examination. None of the goats had any clinical signs of pulmonary disease during the six month observation period. However, at necropsy, grey miliary foci of consolidation (less than 2 mm in size) were seen in the dorsomedial aspects of the diaphragmatic lobes of the lungs in one goat. Light microscopy showed that these were nodular adenomatous formations of tumour acini lined by cuboidal epithelial cells (Fig 1) and intrabronchiolar polypoid growths composed of irregular masses of columnar cells (Fig 2). No lesions were found in the other two goats.

The evidence from this successful transmission of sheep pulmonary adenomatosis to a young goat, judged solely on morphological criteria, demonstrated that the response of the caprine lung to the retrovirus is similar to that of the ovine lung. Since the lesions induced experimentally in sheep are indistinguishable from the naturally occurring disease (Sharp and others 1983, Angus and others 1985) it appears reasonable to assume that any naturally occurring case of caprine pulmonary adenomatosis would have morphological similarities to the experimental lesions described above.

Sheep pulmonary adenomatosis can be transmitted rapidly and with almost 100 per cent efficiency in newborn lambs (Sharp and others 1983). However, the results of the present experiment indicate that transmission of pulmonary adenomatosis to goats, at least with an inoculum derived from sheep, is less efficient and much slower. These observations support some of the prevalence rates which have been reported in goats for the sheep pulmonary adenomatosis-like disease in other countries (Rajya and Singh 1964, Gupta 1967, Tiwari and Pandit 1967) and suggest that the risk of transmission of pulmonary adenomatosis from sheep to goats in the United Kingdom is low.

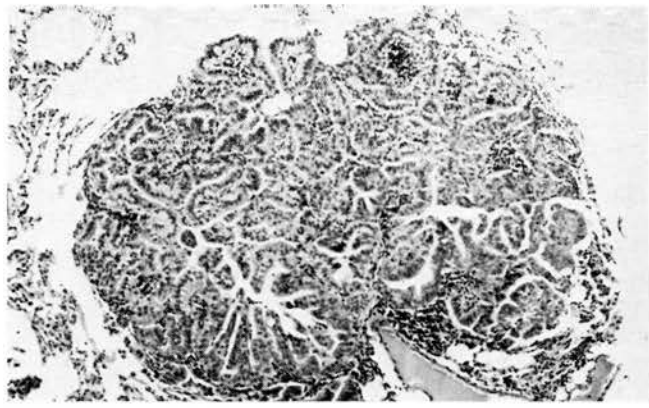

FIG 1: Nodular adenomatous mass in goat lung. The lesion has an acinar arrangement, the acini being lined with cuboidal cells

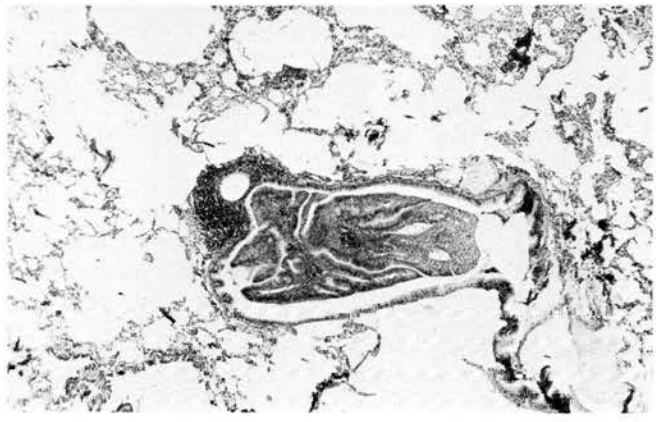

FIG 2: Intrabronchiolar polypoid tumour lesion in goat lung. Haemalum and eosin × 150

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Crystalline composition of equine urinary calculi

EIGHTEEN equine urinary calculi were examined by X-ray diffraction crystallography. The calculi had been recovered from 14 geldings, two stallions and one mare and the site of deposition was the bladder in 14 cases. Calcium carbonate in the forms of calcite plus substituted vaterite was the major component of all the calculi examined. Calcium carbonate crystals were also demonstrated in the urine of two normal horses.

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Successful transfer of N'Dama embryos into Boran recipients

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TEN N'Dama calves raised in Kenya at International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, reached 12 months of age in April 1985. They are the result of a project to transfer frozen N'Dama embryos from The Gambia to Kenya. The project was carried out by ILRAD, the Department of Veterinary Services of Kenya, The Livestock Marketing Board of The Gambia and Granada International Corporation, England.

The objectives of the project were to make disease-free N'Dama cattle available for basic research studies on trypanotolerance (ILCA 1979, Murray and others 1982) in Kenya and, at the same time, to obtain information on the potential use of embryo transfer technology for the exportation of trypanotolerant livestock, the demand for which in west and central Africa greatly exceeds the current supply.

In The Gambia, 26 N'Dama cows were selected as donors and five N'Dama bulls were used for natural service. In accordance with the requirements of the Government of Kenya, donor animals were screened and certified as free from brucellosis and tuberculosis, and the bulls were also free from infection with *Trichomonas fetus* and *Campylobacter fetus*. The Gambia is free from foot-and-mouth disease, rinderpest and enzootic bovine leukosis.

Because of prolonged seasonal anoestrus, it was decided to synchronise donor cows using intravaginal progesterone releasing coils (PRID; Abbot) and to use the PRID-induced oestrus as the starting point for subsequent hormonal programming. Eleven days after the observed oestrus, 16 cows were programmed for superovulation by intramuscular injection of 1500 to 1750 iu of pregnant mare serum gonadotrophin (Folligon; Intervet) followed 48 hours later by an intramuscular injection of 3 ml prostaglandin (Estrumate; ICI).

The remaining 10 cows were programmed by the administration of a total of 28 mg of follicle stimulating hormone (Burns; Biotec) given by intramuscular injection daily over a four day period; 48 hours after the first dose of follicle stimulating hormone, 3 ml of prostaglandin was given. All doses were based on an average cow weight of 230 kg.

On the basis that oestrus would develop within 48 hours of the prostaglandin injection, the cows were placed with a bull on the morning before the day of expected oestrus. No bull had more than two cows in the breeding pens at any one time. Seven days after oestrus/service, the uterus was flushed for embryo removal using a two-way Foley catheter and ovum culture medium (Flow Laboratories). Only 14 of the 26 N'Dama cows had no programming problems and of these seven produced embryos, 30 in all.

Pregnant mare serum gonadotrophin gave better superovulation than follicle stimulating hormone, producing an estimated 4·9 corpora lutea per cow and 4·3 viable ova/embryos per cow, while follicle stimulating hormone gave only an estimated 2·7 corpora lutea per cow and 1·4 viable ova/embryos per cow. One bull proved to be subfertile.

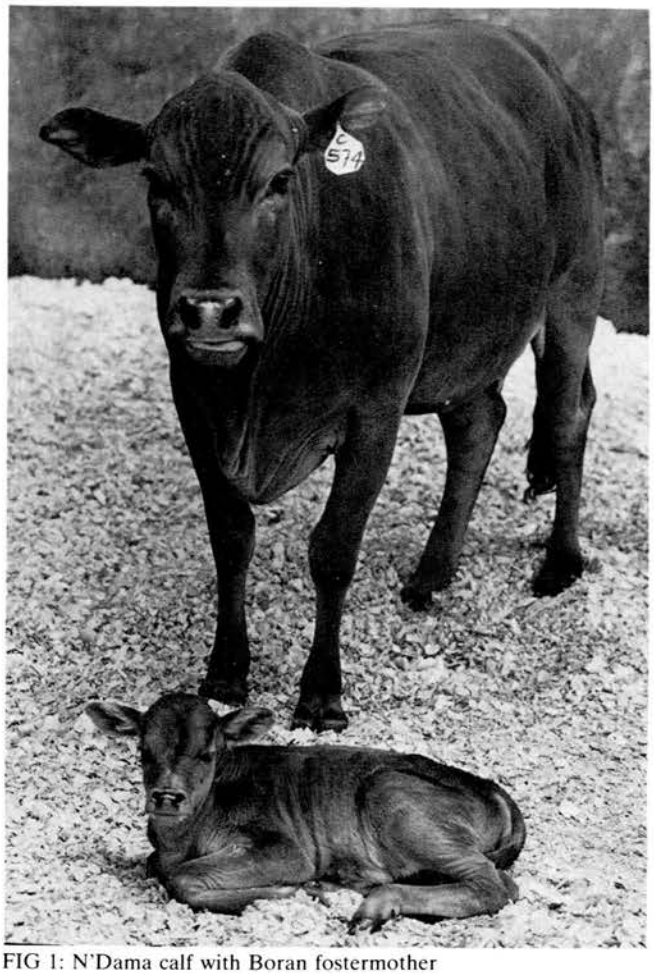

FIG 1: N'Dama calf with Boran fostermother

The embryos were cryopreserved in liquid nitrogen (Trounson and Pugh 1981, Pettit 1985) and, in accordance with animal health import regulations in Kenya, the embryos were held in The Gambia for 30 days before shipment to Kenya.

As prospective recipients, Boran heifers aged 24 to 28 months were selected at Kapiti Plains, the ILRAD ranch in Kenya. Oestrus was synchronised by giving two injections of prostaglandin (2 ml) 11 days apart. Seven days after oestrus, ovaries were assessed per rectum for the presence of a functional corpus luteum and, on this basis, 30 heifers were selected as recipient females.

On the same day (June 20/21, 1983), the frozen embryos were thawed (Trounson and Pugh, 1981, Pettit 1985). One embryo was damaged and discarded with the result that 29 embryos were transferred surgically into the uterine horn ipsilateral to the corpus luteum of 29 Boran heifers. Two months later 11 animals were found to be pregnant on rectal examination. During the sixth month of pregnancy, the pregnant recipients were transferred from Kapiti Plains to ILRAD, Nairobi, in order that they could be monitored more closely. One animal aborted a male calf approximately seven-and-a-half months after implantation; no cause was found.

Between March 23 and April 10, 1984, 10 N'Dama calves, five females and five males, were born with the males being born last. Their birth weights ranged from 16 to 24 kg. No calving problems were encountered and all calves were sucking their 'mothers' within one to two hours of birth. The Boran recipients proved to be excellent foster mothers (Fig 1). After two to three days, the calves were separated from their 'mothers' and reared on whole milk feed. They were weaned at three months of age and at one year of age the females weighed 133±18 kg (mean ± sd) and the males 140±15 kg.

To the authors' knowledge, this is the first time transfer of frozen embryos has been attempted and successfully carried

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Commission of the European Communities

AGRICULTURE

Slow viruses in sheep, goats and cattle

(In particular maedi visna, jaagsiekte, and in caprines, arthritis, encephalitis and pneumonitis)

Proceedings of two workshops, one held in Reykjavik (Iceland) on 13 and 14 July 1982, the other in Edinburgh (Scotland) on 13 and 14 September 1983, sponsored by the Commission of the European Communities, Directorate-General for Agriculture, as part of the research programme on animal pathology

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ISOLATION AND IN VITRO PROPAGATION OF A RETROVIRUS FROM SHEEP PULMONARY ADENOMATOSIS

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Retrovirus particles were first observed in SPA tumour cells by Perk et al (1971) and the association of these particles with the tumour strengthened by biochemical investigations and transmission experiments (Perk et al, 1974; Martin et al, 1976; Sharp et al, 1978; Verwoerd et al, 1980; Herring et al, 1983). However attempts to isolate this retrovirus from the tumour and propagate it in the laboratory have been unsuccessful.

It has been reported that SPA can be induced rapidly in young lambs (Verwoerd et al, 1980; Sharp et al, 1983) and that reverse transcriptase (RT) containing particles can be detected for up to 4 days in cell cultures established from the lung tumours from those lambs (Sharp et al, 1983).

Recently, by means of an immunological technique, we have demonstrated an immunological cross-reaction between the p27 major core polypeptide of Mason-Pfizer monkey virus (MPMV) and a 25000mw polypeptide (p25) in the SPA retrovirus (Sharp and Herring, 1983). In this communication we now describe how this technique has enabled us to demonstrate the replication of the SPA retrovirus for up to 96 days in cell cultures established from tumours induced in young lambs.

The retrovirus present in SPA lung fluids was concentrated by centrifugation (Sharp et al, 1983) and four-fold dilutions titrated in 10 one-day-old lambs by intratracheal inoculation. The lambs were killed by intravenous pentobarbitone when clinical signs of SPA were advanced. So far, only 4 of the 10 lambs have been available for virological examinations. Cell cultures were initiated from tumour tissue of each of the 4 lambs by disaggregation with trypsin, and the supernates assayed for RT activity and SPA retrovirus p25 by immunoblotting (Herring et al, 1983; Sharp and Herring, 1983).

Although RT activity could be detected in the lung fluids obtained from 3 lambs, such activity was demonstrable at low levels on only 3 occasions in cell culture supernates from two lambs (Table 1).

TABLE 1 Detection of SPA retrovirus in supernates of tumour cell cultures from 4 lambs with experimentally induced SPA

Concentration of SPA lung fluid inoculum	lamb	day post-trypsinization on which p25 was detected											
16x	1593				26			50					
	1594	1*											
4x	1597	3*	14	25	32	41*		69	76	82	91	96	
1x	1585	2	16		36	42	57		71				

*day post-trypsinization on which RT activity detected

In contrast, the immunoblotting technique detected the SPA retrovirus p25 in culture supernates from each of the 4 lambs (Fig. 1) and, in 2 cultures, it was demonstrated regularly for up to 96 days during which time the cultures has been passaged 6 times (Table 1). No culture was positive on every occasion on which it was tested. Analysis of the SPA retrovirus, obtained from lung fluid or tumour, by the immunoblotting technique, employing a goat antiserum to MPMV p27, revealed a single polypeptide with a MW of 25,000 and, immediately above it, a broader, indistinct band which we have shown to be the light chain of ovine IgA (data not presented). A notable feature of the immunoblots of the cultures retrovirus is the absence of this IgA band.

Thus we have clear evidence that a retrovirus, possessing several of the known biophysical and antigenic properties of the retrovirus present in SPA lung fluids and tumours, has been isolated and propagated in vitro for up to 96 days. The inability to propagate the SPA retrovirus in vitro has frustrated previous attempts to identify its structural proteins and confirm its role as the aetiological agent. At the moment, it is not apparent whether this cultivated retrovirus can cause SPA. However, we have inoculated it into 3 one-day-old lambs, and the results of this transmission experiment should clarify this point.

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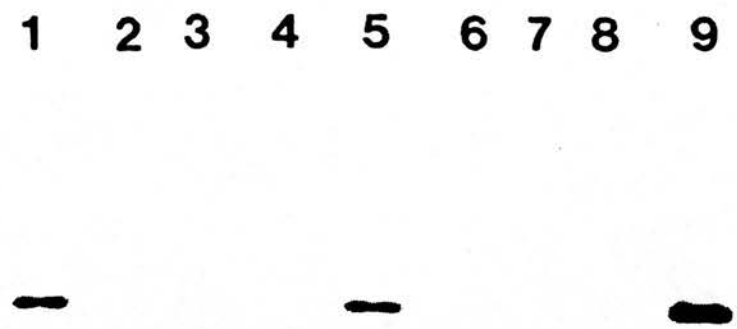

Fig. 1 Detection of p25 in tumour cell culture supernates by a goat antiserum to Mason-Pfizer monkey virus p27 using the Western blotting technique.

lanes 1-3 : supernates from cultures of 1597, 25-41 days post-trypsin
lane 4 : supernate from culture of a natural case of SPA
lanes 5-6 : supernates from cultures of 1585, 16 and 36 days post-trypsinization
lane 7 : supernate from culture of 1593, 26 days post-trypsinization
lane 9 : lung fluid from a natural case of SPA